Long-chain betulaprenol-type polyprenols from the leaves of *Ginkgo biloba*

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A long-chain betulaprenol-type polyprenol mixture was isolated from the leaves of *Ginkgo biloba* mainly as acetate. The structure was determined by mass spectroscopy, $^1$H-n.m.r. spectroscopy and $^{13}$C-n.m.r. spectroscopy. The mixture contained polyprenols-14–22, predominantly polyprenols-17, -18 and -19, and consisted of the dimethylallyl terminal unit (α-terminal), two trans-isoprene residues, a sequence of 11–19 cis-isoprene residues and a terminal hydroxylated isoprene unit (α-terminal) aligned in that order. The concentration of these polyprenols in leaves increased from 0.04 to 2.0% of dry wt. with maturing of the leaves, though the content of total lipids was constant. The distribution of chain length in these polyprenols showed little variation throughout the whole life of the leaves.

Much attention has been directed in recent years towards the nature of dolichols and their derivatives as sugar carriers in the biosynthesis of glycoprotein (Waechter & Lennarz, 1976; Hemming, 1977). Dolichols are a mixture of α-saturated polyprenol homologues consisting of two trans-isoprene residues and different numbers ($m$) of cis-isoprene residues. The alignment of the trans- and cis-isoprene residues was determined as shown in formula (I) (Y. Tanaka & A. Kageyu, unpublished work).

Dolichols of different chain lengths have been isolated from animals (Dallner & Hemming, 1981), plants (Brett & Leloir, 1977) and micro-organisms (Richards & Hemming, 1972; Jung & Tanner, 1973). However, the content of dolichols in the tissues is so small (less than 0.03% of wet wt.), except for human internal organs (0.01–0.3% of wet wt.) (Rupar & Carroll, 1978), that it is difficult to obtain a sufficient amount of dolichols for comprehensive investigations.

Several types of polyprenols have been found in the plant kingdom during the past three decades. Most of the polyprenols isolated from the leaf tissues of angiosperms have consisted of three internal trans-isoprene units and all other residues including α-terminal unit in the cis-configuration. The alignment of the cis- and trans-isoprene residues in ficaprenol-11 isolated from *Ficus elastica* was determined by $^{13}$C-n.m.r. spectroscopy to be as shown in formula (II) ($m = 7$) (Tanaka & Takagi, 1979).

In contrast with the ficaprenols, only two examples containing two trans-isoprene residues (III) are recognized in polyprenols: betulaprenols-6–9 ($m = 3–6$) from the woody tissue of *Betula verrucosa* (Wellburn & Hemming, 1966b), and bacterial polyprenol-11 ($m = 8$) from *Lactobacillus plantarum* (Gough et al., 1970). The content of these polyprenols is fairly small (0.004–0.03% of wet wt.). Although the chain lengths of these polyprenols are shorter than those of mammalian dolichols, the fundamental alignment of the trans- and cis-isoprene residues is expected to be identical with that of dolichols, except for the absence of the saturation in the α-terminal residue.

We decided to undertake a series of investigations to search for more abundant sources of polyprenols of betulaprenol-type with $m > 10$ as starting materials for the synthesis of mammalian dolichols. The present paper reports the isolation and the structural characterization of such polyprenols from the leaves of *Ginkgo biloba*. Polyprenols consisting of two trans-isoprene residues and 11–19 cis-isoprene residues were obtained in a high yield, up to 2% of dry wt. of leaves. These polyprenols were found to have the same alignment of the trans- and cis-isoprene residues as that of mammalian dolichols.

Methods

Isolation and purification of polyprenols

The leaves were collected from the same *Ginkgo biloba* tree (about 30 years old, male) seven times at
CH₃C=CC₂C=CH₂ \[ \text{CH₃C=CC₂C=CH₂} \] \[ \text{CH₃C=CC₂C=CH₂} \] \[ \text{CH₃C=CC₂C=CH₂} \] (I)

CH₃C=CH₂ \[ \text{CH₃C=CH₂} \] \[ \text{CH₃C=CH₂} \] \[ \text{CH₃C=CH₂} \] (II)

CH₃C=CH₂ \[ \text{CH₃C=CH₂} \] \[ \text{CH₃C=CH₂} \] \[ \text{CH₃C=CH₂} \] (III)

intervals of about 1 month after bud unfolding until leaf shedding. These leaf samples (each 200 g) were dried for 1–2 days at 50–60°C in an air-circulating drying oven. The dried leaves (each 50 g) containing 5–9% moisture were crushed into pieces of about 5 mm diameter and extracted three times with 600 ml of solvent for 3–9 days for each extraction at room temperature (approx. 20°C). The most effective extraction was performed with acetone/n-hexane (1:1, v/v). Lipids (3.814 g) containing 0.900 g of polyprenyl acetates (1.97% of dry wt. of leaves) were obtained from the leaves harvested in November.

The lipid extract was subjected to column chromatography on silica gel NW-6201 (Yamani Chemicals, Osaka, Japan) packed into a 30 mm-diameter column. Elution with n-hexane/diethyl ether (19:1, v/v) gave 1.243 g of polyprenyl acetate fraction (purity 72%), which had Rₓ 0.41 on t.l.c. on silica gel 60 F₂₅₄ plates (Merck, thickness 0.25 mm) developed with n-hexane/ethyl acetate (19:1, v/v). Elution with n-hexane/diethyl ether (17:3, v/v) gave 0.251 g of polyprenol fraction (purity 7%), which had Rₓ 0.44 on t.l.c. on the same silica gel 60 F₂₅₄ plate developed with n-hexane/ethyl acetate (17:3, v/v). Then 0.934 g of polyprenyl acetate fraction (purity 96.4%) was obtained by gel-permeation chromatography on a preparative column (21.2 mm internal diam. × 600 mm) packed with high-resolution styrene/divinylbenzene gel (Tanaka et al., 1982a), with chloroform as eluent. The isolated pale-yellow oily substance was dissolved into about a 20-fold volume of acetone, and 0.015 g of waxy precipitate was filtered off. The 0.900 g amount of pure polyprenyl-14–22 acetates (and a trace amount of polyprenyl-23 acetate) was further fractionated into each component by using a reversed-phase high-pressure liquid-chromatography column (10 mm internal diam. × 300 mm) packed with Nucosil 5C₁₈ (Macherey-Nagel) with acetone/methanol (9:1, v/v) as eluent at a flow rate of 3 ml/min monitored by a Knauer 98.00 differential refractometer.

Fig. 1. Reversed-phase high-pressure liquid chromatography of polyprenyl acetates
For experimental details see the text.

leosil 5C₁₈ (Macherey–Nagel) with acetone/methanol (9:1, v/v) as eluent at a flow rate of 3 ml/min monitored by a Knauer 98.00 differential refractometer.
Measurements

Mass spectra were determined in the field-desorption mode with a JEOL JMS D-300 gas chromatograph-mass spectrometer. Perfluoropoly-(propylene oxide) was used as a reference compound for the calibration of mass numbers. ^1H-n.m.r. and ^13C-n.m.r. spectra were obtained with JEOL FX-200 and GX-400 spectrometers at 200 MHz and 400 MHz for ^1H n.m.r. and 50.1 MHz and 100.5 MHz for ^13C n.m.r. Measurements were made in C\textsubscript{2}HCl\textsubscript{3}, with tetramethylsilane as an internal standard, at room temperature. I.r. spectra were obtained with a Digilab FTS-200 C/D Fourier-transform infrared spectrometer. Measurements were made for the samples in KBr discs at room temperature.

Results and discussion

Distribution of chain length

Fig. 1 shows the distribution of chain length in the polypropenyl acetate mixture determined by reversed-phase high-pressure liquid chromatography. The field-desorption mass spectrum of the same mixture showed a similar distribution of molecular ions corresponding to the distribution of chain length as shown in Fig. 2. The mass numbers (m/z) are reasonably assigned to the molecular ions of polypropenyl-14--22 acetates; theoretical values are 1012, 1080, 1148, 1216, 1284, 1352, 1420, 1488 and 1556 respectively. Saponification products from the polypropenyl acetate mixture gave a field-desorption mass spectrum with peaks at 970, 1038, 1106, 1174, 1242, 1310, 1378, 1446 and 1514, which are in accord with the theoretical values for polypropenols-14--22. Therefore the peaks in Fig. 1 are reasonably assigned to polypropenyl-14--22 acetates (and a small peak due to polypropenyl-23 acetate), which were confirmed by field-desorption mass-spectral measurements of the isolated polypropenyl acetates and the polypropenols obtained by saponification thereof. The percentages by weight of polypropenyl-14--23 acetates were found to be 0.9, 1.6, 6.5, 24.9, 36.5, 17.7, 6.7, 2.9, 1.6 and 0.8%, the average number of isoprene units being 17.9, from the peak areas in Fig. 1. Here, it was checked that the weight fractions of polypropenyl acetates were directly proportional to the peak areas of high-pressure liquid chromatograms, with a maximum
deviation from linearity of ±3%. These findings indicate that the polyprenyl acetates from the leaves of Ginkgo biloba are a mixture of homologues with a distribution of chain length similar to that of mammalian dolichols.

**Structural characterization**

Polyprenyl-18 acetate isolated from the polyprenyl acetate mixture showed i.r.-absorption bands characteristic of cis-isoprene residues and acetate, which is in good agreement with previously reported spectra (Burgos et al., 1963; Wellburn et al., 1967). The other polyprenyl acetates gave essentially the same spectra.

Fig. 3 shows the $^1$H-n.m.r. spectrum of polyprenol-18 obtained by saponification of polyprenyl-18 acetate. The spectrum is in agreement with those of cis-trans-polyprenols (Feeney & Hemming, 1967; Stone et al., 1967; Wellburn et al., 1967); the signals at 1.68 and 1.60 p.p.m. are assigned to the methyl protons of the internal cis- and trans-isoprene residues respectively. Both signals also include those due to the methyl protons of the ω-terminal unit in the cis- and trans-configurations. A small signal at 1.74 p.p.m. is assigned to the methyl protons of the α-terminal cis-isoprene unit. The other polyprenols showed the same signals, indicating that the α-terminal cis-isoprene unit is a common structure for these polyprenols. The observed relative intensities are in good agreement with calculated values determined on the assumption of the presence of two internal trans-isoprene residues for each polyprenol, as listed in Table 1. These findings demonstrate that the polyprenols are a series of homologues consisting of two internal trans-isoprene residues and different numbers of internal cis-isoprene residues.

The alignment of the internal cis- and trans-isoprene residues can be determined by the $^{13}$C-n.m.r. method (Tanaka et al., 1982b). Polyprenol-18 showed the same $^{13}$C-n.m.r. signals as ficaprenol-11 (Tanaka & Takagi, 1979), as shown in Fig. 4(a). The signals are assigned by the comparison of the chemical shifts with those of model compounds (Tanaka et al., 1982b), and also by the application of INEPT (insensitive nuclei enhanced by polarization transfer) $^{13}$C-n.m.r. measurement (Doddrell & Pegg, 1980), as listed in Table 2. The C(1)-methylene carbon atoms exhibited three signals around 32–40 p.p.m., reflecting the linkage of the cis-
Long-chain betulaprenol-type polyprenols from the leaves of *Ginkgo biloba*

Table 1. Relative intensities of $^1$H-n.m.r. signals in polyprenols-15–20
Theoretical values are in parentheses. For experimental details see the text.

<table>
<thead>
<tr>
<th>Chemical shift (p.p.m.)</th>
<th>Assignment</th>
<th>Number of isoprene units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.60</td>
<td>$CH_3;trans$</td>
<td>15 16 17 18 19 20</td>
</tr>
<tr>
<td>1.61</td>
<td>$CH_3;trans(\omega)$</td>
<td>(3) (3) (3) (3) (3) (3)</td>
</tr>
<tr>
<td>1.68</td>
<td>$CH_3;cis,cis(\omega)$</td>
<td>11.9* 13.0* 14.1* 14.9* 16.1* 17.0*</td>
</tr>
<tr>
<td>1.74</td>
<td>$CH_3;cis(\alpha)$</td>
<td>1.08* 0.93* 0.97* 0.98* 0.96* 1.03*</td>
</tr>
<tr>
<td>4.08, 4.10</td>
<td>$CH_2OH$</td>
<td>1.84 1.97 1.96 1.92 1.96 2.05</td>
</tr>
<tr>
<td>5.12</td>
<td>$=CH$</td>
<td>14.0 15.0 16.0 17.0 18.0 18.9</td>
</tr>
<tr>
<td>5.42, 5.44, 5.46</td>
<td>$=CH-CH_2OH$</td>
<td>1.12 1.03 1.07 1.06 1.01 1.00</td>
</tr>
</tbody>
</table>

* The observed and theoretical values for the methyl protons are the number of methyl groups.

Fig. 4. $^{13}$C-n.m.r. spectrum of polyprenol-18 (a) and expanded spectrum of $C_{(1)}$-methylene carbon signals (b)
For experimental details see the text.

and trans-isoprene residues, as shown in Fig. 4(b), where the carbon atoms are designated as follows:

$$C_{(3)} \ \ C_{(1)}$$
$$-C_{(1)}-C_{(2)}=C_{(3)}-C_{(4)}$$

The signal at 39.78 p.p.m. is assigned to the $C_{(1)}$-methylene carbon atoms of the trans-isoprene residue in the trans–trans- and $\omega$–trans-linkages. The signals at 32.29 and 32.05 p.p.m. are assigned to the $C_{(1)}$-methylene carbon atoms of the cis-isoprene residue in cis–cis- and trans–cis-linkages respec-
Table 2. Assignment of \(^{13}\)C-n.m.r. signals in polypropenols-15–20

For experimental details see the text. The carbon atoms are designated as follows:

<table>
<thead>
<tr>
<th>Chemical shift (p.p.m.)</th>
<th>Assignment</th>
<th>Chemical shift (p.p.m.)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.99</td>
<td>5-trans</td>
<td>124.28</td>
<td>3-(\omega), 3-(\alpha)</td>
</tr>
<tr>
<td>17.65</td>
<td>5-(\omega)</td>
<td>124.32</td>
<td>3-trans</td>
</tr>
<tr>
<td>23.42</td>
<td>5-cis, 5-(\alpha)</td>
<td>124.52</td>
<td></td>
</tr>
<tr>
<td>25.65</td>
<td>1-(\omega)</td>
<td>124.62</td>
<td></td>
</tr>
<tr>
<td>26.49</td>
<td>4-cis</td>
<td>124.75</td>
<td></td>
</tr>
<tr>
<td>26.71</td>
<td>4-trans</td>
<td>124.98</td>
<td>3-cis</td>
</tr>
<tr>
<td>26.87</td>
<td>4-(\omega)</td>
<td>125.12</td>
<td></td>
</tr>
<tr>
<td>32.05</td>
<td>1-trans–cis</td>
<td>131.04</td>
<td>2-(\omega)-trans</td>
</tr>
<tr>
<td>32.29</td>
<td>1-cis–cis</td>
<td>134.85</td>
<td>2-trans–trans</td>
</tr>
<tr>
<td>39.78</td>
<td>1-trans–trans</td>
<td>135.15</td>
<td>2-cis</td>
</tr>
<tr>
<td>58.99</td>
<td>4-(\alpha)</td>
<td>135.31</td>
<td>2-trans–cis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>135.96</td>
<td>2(\alpha)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>139.56</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Relative intensities of \(^{13}\)C-n.m.r. signals in polypropenols-17–19

For experimental details see the text. Integrated relative intensities were obtained by gated decoupling \(^{13}\)C-n.m.r. measurement. Theoretical values are in parentheses.

<table>
<thead>
<tr>
<th>Chemical shift (p.p.m.)</th>
<th>Assignment</th>
<th>Number of isoprene units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>32.05</td>
<td>trans–cis</td>
<td>0.96 (1)</td>
</tr>
<tr>
<td>32.29</td>
<td>cis–cis</td>
<td>13.0 (13)</td>
</tr>
<tr>
<td>39.78</td>
<td>trans–trans, (\omega)-trans</td>
<td>2.09 (2)</td>
</tr>
</tbody>
</table>

Tatively (Tanaka et al., 1982b). The absence of the signal around 40.0 p.p.m., which is characteristic of the cis–trans-linkage, indicates that the trans-isoprene units are incorporated in the \(\omega\)-trans–trans-linkage. The presence of the \(\omega\)-trans-linkage is also confirmed by the characteristic \(\text{C}_{21}\)-carbon signal of the \(\omega\)-terminal unit at 131.04 p.p.m.; the \(\omega\)-trans-linkage in model compounds showed a signal around 131.0–131.3 p.p.m., and the \(\omega\)-cis-linkage a signal around 131.5–131.6 p.p.m. (Tanaka et al., 1983). The relative intensities of the signals reflecting the trans–trans, \(\omega\)-trans, cis–cis, and trans–cis linkages were determined for polypropenols-17, 18 and 19, as listed in Table 3. Here, gated decoupling measurement was applied in order to eliminate the nuclear Overhauser enhancement factor, and also the spectra were obtained with multiple scans at a pulse repetition time of 20s, considering the spin–lattice relaxation times (\(T_1\)) of 1.2 s (\(C_{11}\) in trans-isoprene unit) and 0.6 s (\(C_{11}\) in cis-isoprene unit). The observed values are in good agreement with those expected for the alignment of the \(\omega\)-terminal isoprene residue, two trans-isoprene residues and 14–16 cis-isoprene residues aligned in that order. From these findings and the content of trans-isoprene residues in other polypropenols, it can be concluded that the polypropenols-15–20 have the structure (III).

Seasonal variation

The seasonal variation of the content of total lipids, polypropenyl acetates and polypropenols extracted from Ginkgo biloba leaves is shown in Fig. 5. Whereas the amount of total lipids was nearly constant throughout the season, a remarkable increase was observed for the content of polypropenyl...
Fig. 5. Seasonal variation of concentrations (% of dry wt. of leaves) of total lipids (△), polyprenyl acetates (○) and polyprenols (□) with the age of Ginkgo biloba leaves. For experimental details see the text.

acetates with the maturing of the leaves. Similar observations have been reported for Aesculus hippocastanum and several other higher plants (Wellburn & Hemming, 1966a). The curve for polyprenyl acetates demonstrates the presence of two critical points, the first in early summer and the second in late autumn. The ultimate content of 2.0% of dry wt. of leaves was observed in late autumn. On the other hand, the distribution of chain length was essentially the same average length of isoprene residues of 17.7 ± 0.3 from the budding to leaf shedding, as in the case of castaprenols (Wellburn & Hemming, 1966a). The constancy of the distribution pattern was also observed for several other samples from Ginkgo biloba having different age, sex and climate where the tree has grown.

The synthesis of ‘synthetic’ dolichol was attempted by using the polyprenyl acetates as the starting material, and the ‘synthetic’ dolichol showed the same distribution of chain length as that extracted from pig liver (S. Suzuki, F. Mori, T. Takigawa, K. Ibata, Y. Ninagawa, T. Nishida, M. Mizuno & Y. Tanaka, unpublished work).

References