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ALKANETRIOLS IN PSILOTOPHYTE CUTINS*

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Key Word Index—*Psilotum*; *Tmesipteris*; Psilotophyte; cutin; hydroxy-fatty acids; hexadecanetriols; TMSi ethers; high resolution mass spectrometry.

Abstract—The covalently bonded components of the stem cutin of *Psilotum* include 16-hydroxyhexadecanoic acid and substantial amounts of hexadecane-1,8,16-triol. While of generally similar composition, leaf cutin of *Tmesipteris* contains a mixture of hexadecanetriol isomers. The findings suggest that psilotophyte cutins evolved in a different manner from those of other land plants.

INTRODUCTION

Chemical studies of plant cutins have been concerned mainly with angiosperms [1–5] and, to a lesser extent, with the gymnosperms and lower plants [6–8]. The lipids released upon hydrolysis of cutins are almost exclusively hydroxy-fatty acids. Only rarely long chain alcohols occur, and even then only in trace amounts [5–7].

In this study of the only extant psilotophyte genera, solvent-extracted cutins from the stems of *Psilotum nudum* and the leaves of *Tmesipteris vieillardii* have been found to contain substantial proportions of bound hexadecanetriols.

RESULTS

Psilotum cutin

The IR spectrum (KBr disc) of *Psilotum nudum* stem cutin revealed aliphatic $\underline{\nu}$ CH (2930, 2860 cm^{-1}), ester $\underline{\nu}$ C=O (1735 cm^{-1}) as well as $\underline{\nu}$ OH (~3600 cm^{-1}) and minor aromatic $\underline{\delta}$ CH (1600 cm^{-1}) vibrations. The cutin was inert to 3 N mineral acid (30 hr at 100°) and 11 N HCl saturated with $ZnCl_2$ (100° for 10 min), but on reflux with alcoholic KOH yielded *ca* 27% ether-soluble lipids (see Table 1). This ether extract includes substantial amounts of hexadecane-1,8,16-triol (*ca* 10·3% of the cutin). TLC, micro-IR, micro-PMR, and high resolution mass spectrometry (HRMS) of its Tris-TMSi ether are in accord with this assignment. The structure was initially inferred from GC–MS analysis of the derivatized lipid fraction and confirmation came from HRMS analysis of the TMSi derivative, prepared from a single component isolated from the non-saponifiable fraction by TLC and preparative GLC. The resulting HRMS data are comparable to the low resolution GC–MS data.

The MS fragmentation pattern of the compound identified as 1,8,16-Tris-TMSioxyhexadecane, derived from *Psilotum* cutin hydrolysate is summarized in Fig. 1. All the ion compositions were confirmed by HRMS measurements and some fragmentation pathways are corroborated by metastable ions. The major MS fragmentation observed gives rise to α-cleavage ions containing two Si atoms. Indeed, the ion of composition $C_{14}H_{33}O_2Si_2$ at *m/e* 289 is identical to that observed in the disilyl α-cleavage from methyl di-TMSioxyhexadecanoate. As expected, the disilyl-ions at *m/e* 289 and 303 expel trimethylsilanol, giving rise to ions $C_{11}H_{23}\dot{O}Si$ at *m/e* 199 and $C_{12}H_{25}\dot{O}Si$ at *m/e* 213. The high-mass region exhibits the ion $C_{24}H_{55}\dot{O}_3Si_3$ at *m/e* 475, derived by loss of a methyl radical from a very weak M^+.

* Part 5 in the series Gas Chromatographic–Mass Spectrometric studies of Long Chain Hydroxy Acids, for part 4 see D. H. Hunneman and G. Eglinton, (1972) *Phytochemistry* **11**, 1989.

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Table 1. Cutin hydrolysis products of psilotophytes

Compound (as methyl ester, TMSi ether derivatives)	*Psilotum nudum* (stem) Ether soluble (%)*	*Tmesipteris vieillardii* (leaf) Ether soluble (%)*	*Tmesipteris vieillardii* (leaf) $NaHCO_3$ treated, Et_2O soluble (%)	Retention index† OV-1 SE-30†
8-Hydroxyoctanoic acid	0·7	—	—	1480†
9-Hydroxynonanoic acid	1·2	—	—	1580†
m-Coumaric acid	1·3	—	—	1670†
Unidentified phenolic acid	—	1·9	—	1730
p-Coumaric acid	1·2	—	—	1785†
12-Hydroxydodecanoic acid	—	2·7	—	1880
Hexadecanoic acid	0·4	3·1	6·0	1920
Unidentified phenolic acid	—	1·2	—	1940
Tetradecane-1,14-dioic acid	—	3·4	—	2030
14-Hydroxytetradecanoic acid	0·3	28·5	48·0	2080
Octadecanoic acid	—	4·3	—	2120
Hexadecane-1,16-dioic acid	—	1·9	—	2230
16-Hydroxyhexadecanoic acid	52·8	17·1	24·0	2280
Eicosanoic acid	—	3·7	—	2320
18-Hydroxyoctadecenoic acid	0·9	—	—	2460†
18-Hydroxyoctadecanoic acid	1·1	5·5	—	2480
Hexadecane-1,8,16-triol‡	38·3	3·8	5·5	2520
Hexadecane-1,7,16-triol‡	—	3·1	4·5	2520
Hexadecane-1,6,16-triol‡	—	2·3	3·5	2520
Hexadecane-1,5,16-triol‡	—	0·8	1·2	2520
Hexadecane-1,4,16-triol‡	—	0·2	0·3	2520
Docosanoic acid	—	5·0	—	2520
Remaining unknown peaks	2·4	11·5	7·0	—

* % Lipids.

† GLC conditions: 250 cm × 1·6 mm stainless steel column, packed with Gas Chrom Q coated with 1–2% either OV-1 or SE30, and using N_2 at a flow rate of 15 ml/min.

‡ Quantitative estimates obtained from intensity of relevant α-cleavage fragment ion pairs and γ-90 fragments, e.g. *m/e* 289/303; *m/e* 275/317; *m/e* 261/313.

The GC-MS and HRMS data exhibit the low-mass ions C_3H_9Si at *m/e* 73 and $C_4H_{11}SiO$ at *m/e* 103, indicating monosilyl cleavage.

Additionally, novel rearrangement ions observed in the high mass region, involve the expulsion of an alkene moiety (routes A and B, Fig. 1). The ion $C_{16}H_{39}O_3Si_3$ at *m/e* 363 decomposes to a trisilyl fragment $C_7H_{21}O_2Si_3$ at *m/e* 221. The metastable for the respective transition of its apparently related *m/e* 377 ion ($C_{17}H_{41}O_3Si_3$) is somewhat obscured. In the MS data of the synthetic 1,7,16-isomer, analogous ions were observed at *m/e* 349 and 391 [8], of which the *m/e* 349 ion ($C_{15}H_{37}O_3Si_3$) was confirmed by HRMS to be in the same $(C_nH_{2n+7}O_3Si_3)^+$ series. The analogous, though less complex, expulsion of an alkene fragment from the M-15 ions of 1-phenyl-2-trimethylsilylethane has been reported previously [9]. The *m/e* 221 ion may well be of special structural significance in the MS of Tris–TMSi ethers generally. No previous structural information has been deduced from this ion, though it was observed by Eglinton *et al.* [10] in the MS of silicate TMSi ethers. Esselman and Clagett [11] and Bryce *et al.* [12] do not confirm the presence of rearrangement ions where an alkene moiety is expelled from the $\dot{M}$-15 ion in MS of poly-TMSoxy alkanes. Therefore it appears that this specific type of rearrangement ion requires an isolated secondary TMSi function in the presence of primary TMSi groups.

The α-cleavage ions *m/e* 303 and 289 are about equal in relative intensity, as is the case for the rearrangement ions. *m/e* 363 and 377. Both halves of the molecule about the secondary OTMSi function are nearly identical and the probabilities of the fragment ion formation are similar as there is no apparent restriction in the flexibility of the system. Indeed, the flexibility of excited aliphatic systems in the vapour state has previously been

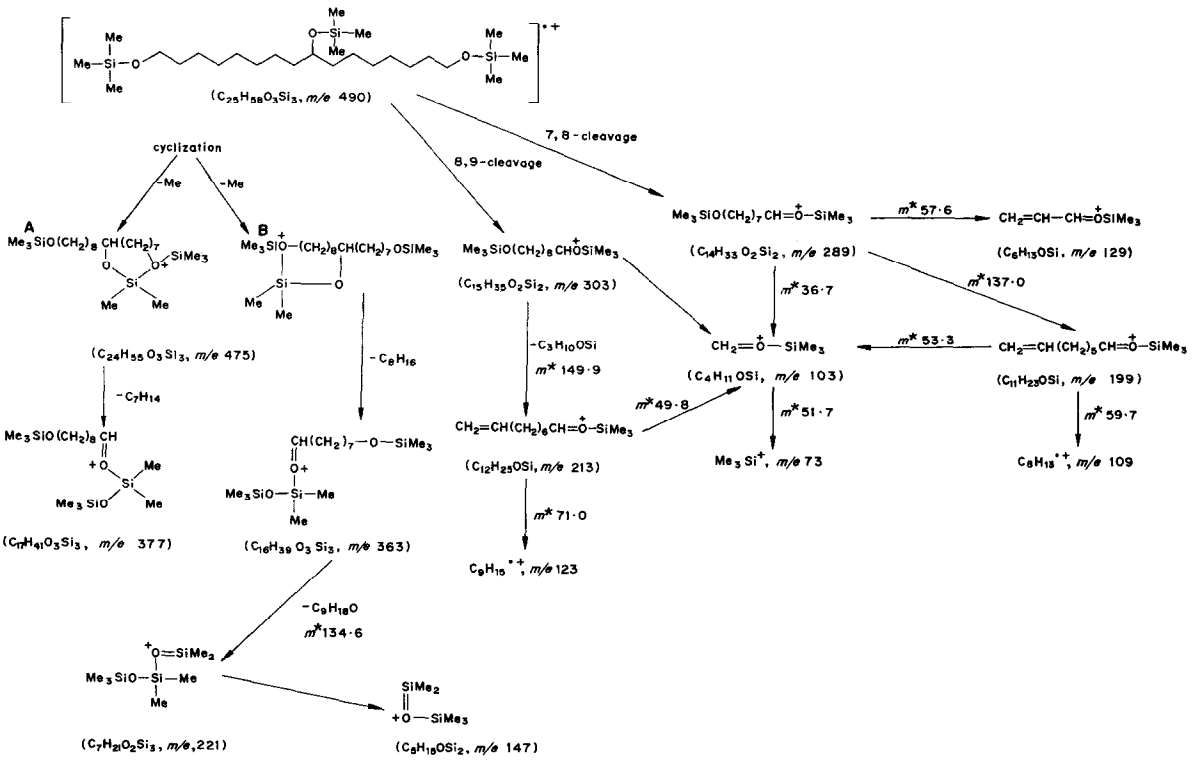

Fig. 1. Proposed MS fragmentations of 1,8,16-tris-TMSoxyhexadecane [8]. All ion compositions were confirmed by HRMS.

considered important, both in the MS migration of the TMSi function [13] and in the formation of macrocyclic ions of the M-15 type [14].

Additional structural confirmation was provided by the synthesis of the isomeric 1,7,16-Tris-TMSioxyhexadecane from methyl 10,16-dihydroxyhexadecanoate by reduction with $LiAlH_4$. The reduction product, which contained minor amounts of the 1,8,16-isomer derived from traces of methyl 9,16-dihydroxyhexadecanoate present in the starting material, was shown to possess identical GLC, and closely similar GC–MS characteristics to the *Psilotum* triol (as the TMSi derivative). HRMS carried out on this product confirmed the compositional identity of the ions. Walton and Kolattukudy [5] in their hydrogenolysis studies of cutin have published MS of both deuterated and non-deuterated Tris-TMSioxyhexadecanes. Their results are also in agreement with the present findings.

The structure of the major lipid constituent, isolated from the saponifiable fraction, 16-hydroxyhexadecanoic acid (Table 1), was confirmed by GC–MS and micro-IR analysis of the TMSi ether, methyl ester. Minor amounts of 8-hydroxyoctanoic and 9-hydroxynonanoic acids were identified among the saponification products by GC–MS. These homologues have not been reported previously in cutin hydrolysates of higher plants.

Tmesipteris cutin

Tmesipteris vieillardii cutin appeared to be heavily impregnated by tannin-like pigments. The IR spectrum suggested a predominance of alkyl and ester groups in addition to minor hydroxyl and aromatic moieties. Saponification of the cutin gave rise to approximately 25% ether-soluble lipids (Table 1) in which 14-hydroxytetradecanoic and 16-hydroxyhexadecanoic acids are major constituents. Minor components of the hydrolysate include tetradecane-1,14-dioic and hexadecane-1,16-dioic acids, as well as phenolic acids.

A further portion of the cutin was exhaustively washed with aqueous bicarbonate solution, whereupon the associated tannins were largely removed from the cutin. GC–MS analysis

revealed that bicarbonate treatment of *Tmesipteris* cutin extracted minor amounts of fatty acids, as well as tannins. Subsequent saponification of this bicarbonate-leached cutin released mainly fatty acids, ω-hydroxy-fatty acids and alkanetriols (Table 1).

Of particular interest was the presence of a mixture of five isomeric hexadecanetriols in the saponification products. The α-cleavage ions in the LRMS data of *Tmesipteris*-derived Tris–TMSioxyhexadecanes were observed as pairs at *m/e* 303/289; 275/317; 261/331; 247/345 and 233/359. In the case of the 261 ion the decomposition ion at *m/e* 171 resulting from expulsion of trimethylsilanol is particularly intense, compared with other ions in the series. A similar phenomenon is observed in the mass spectrum of methyl 10,15-*di*-TMSioxypentadecanoate [6]. The rearrangement ions resulting from the expulsion of an alkene fragment from the $\dot{M}$-15 ion is similarly observed in the isomeric *Tmesipteris*-derived Tris–TMS-oxyhexadecanes. Further corroboration of their structures is provided by successful GLC co-injection with semi-synthetic 1,7,16- and 1,8,16-Tris–TMSioxyhexadecanes on OV-210 and OV-1 GLC phases, yielding single unresolved peaks.

In all Tris–TMSioxyhexadecane MS reported herein, the ions *m/e* 147 and 149 are present. These ions, especially the former, are widespread in compounds containing more than one TMSi function [3].

DISCUSSION

The psilotophytes examined possess well-developed, but atypical cutins (Table 1). It seems likely that they evolved differently from mosses, lycopods, ferns, gymnosperms and angiosperms [6,8]. All previously studied cutins contain only traces of monohydric or dihydric alcohols. Dihydroxyhexadecanoic, trihydroxyoctadecanoic, or 9,10-epoxy-18-hydroxyoctadecanoic acids, which are usually present as major constituents [1–8] were absent from *Psilotum* and *Tmesipteris* cutins.

Tse and Towers [15] report a 6-phenylcoumalin glycoside, psilotin, found only in the psilotophytes. Thus, the novel cutin lipid, hexadecane-1,8,16-triol and its related 1,4,16-, 1,5,16-, 1,6,16- and 1,7,16- isomers do not represent the only natural products apparently confined to this group of plants.

Certain plant waxes contain oligomeric esters consisting entirely of ω-hydroxy alkanoic acids of chain length C_{12}-C_{16} [16]. It has been thought that these estolides and the dihydroxyhexadecanoic acid-rich cutins may be genetically related. Similarly, some plant waxes contain oligomeric esters of ω-hydroxy alkanoates and α,ω-alkenediols [17]. If the former hypothesis is correct, then a parallel relationship between this latter ester-type and the psilotophyte cutins would seem reasonable.

The positions of the in-chain hydroxyl group in the more abundant *Tmesipteris*-derived hexadecanetriols are similar to those of the dihydroxyhexadecanoic acids derived from most plant cutins. This observation and the available biosynthetic data for dihydroxyhexadecanoic acids in cutins [3] and for nonacosan-15-ol [18] in plant waxes make it highly probable that the secondary hydroxyl of the psilotophyte hexadecanetriols is introduced by a direct hydroxylation step (Fig. 2). If pathway "B" operates the psilotophytes will possess a further enzyme in the C_{16} cutin-synthe-

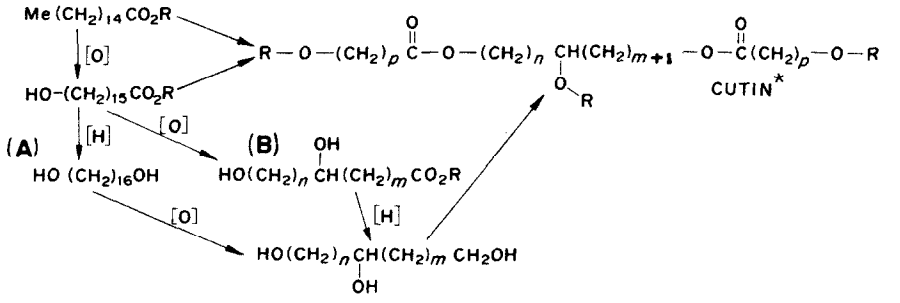

Fig. 2. Proposed hypothetical biosynthesis of *Psilotophyte* cutin. R = Glycoside and/or phenolic (probably polymeric), $n + m = 14$, $p = 15$ and/or 13. (* Cutin based on an analogous estolide identified by Kariyone *et al* [17].

sizing pathway, which renders them more "advanced" than all other cutin-bearing plants so far studied. If pathway "A" operates, the cutin-synthesizing pathway will have evolved in a substantially different manner from most other C_{16} cutin-containing plants by utilising hexadecanediol as a cutin precursor. In view of the known distribution of hexadecanediols in nature as bound lipid constituents, this latter hypothesis seems more reasonable. A study of cutin-bound hexadecanetriol biogenesis in the psilotophytes would hence prove rewarding from the evolutionary aspect. Further, it seems probable that the isomeric hexadecanetriols are biosynthesised by a number of closely-related hydroxylases. In view of the observed polyploidy in the genus *Tmesipteris* [19], mutation of the hypothetical hydroxylase gene might well account for the formation of the series of isomers [20]. Indeed, a number of genetically-related hydroxylases may also account for the range of dihydroxyhexadecanoate isomers present in many cutins. Whether the alkanetriol component of *Psilotum* cutin represents a deleto-mutation of numerous hypothetical hydroxylases of the *Tmesipteris* type, or whether these never arose in the first instance, remains uncertain.

Long-chain polyhydroxy alcohols are of limited occurrence in nature, and are usually bound in some way. A series of alkane-1,2,3,4-tetraols has been reported in a plant resin [21]. Recently Bryce *et al.* [12] have isolated and partially characterised the *O*-hexoside of hexacosane-1,3,25-triol from the heterocysts of the blue–green alga *Anabaena cylindrica*. Other than those herein reported, this is the only long-chain aliphatic trihydric alcohol known to occur in plants.

The presence of 18-hydroxyoctadecanoic and 18-hydroxyoctadecenoic acids in *Psilotum* cutin hydrolysate provides a link between the psilotophyte, gymnosperm and angiosperm taxa. In the psilotophytes these acids appear, however, to be unaccompanied by epoxy, dihydroxy and trihydroxy analogues, in which respect the psilotophyte cutins do differ from those of gymnosperms and angiosperms [3,6,8]. The 8-position of the secondary hydroxyl in 1,8,16-trihydroxyhexadecane derived from *Psilotum nudum* (Table 1) is similar to that of the dihydroxyhexadecanoic acid fraction from *Pinus silvestris* cutin hydrolysate. According to Hunneman's hypothesis [7], the above would suggest that Pinales and Psilotales share a common ancestor. The cutin hydrolysate of *Tmesipteris*, however, does provide conflicting evidence, due to the presence of a large number of related isomers (Table 1).

The minor amounts of phenolic constituents in psilotophyte cutin hydrolysates may, on comparison with findings by Vandenburg and Wilder [22], be part of the cutin structure. Small amounts of phenolic acids were found in an estolide wax [22], and were presumed to be chemically bound to the wax, probably as esters. The nature of phenolic bonding to cutin is difficult to establish although phenolics are readily extracted by mild alkali ($NaHCO_3$) treatment of *Tmesipteris* cutin (Table 1). The substantial amounts of tannins in *Tmesipteris* cutin may well provide the plant with additional protection against pathogenic fungi [23]. The unusual occurrence of C_8 and C_9 ω-hydroxy fatty acids (Table 1), while interesting, cannot be ascribed any particular taxonomic significance.

EXPERIMENTAL

HRMS analyses were carried out on an AEI-MS 902 high resolution mass spectrometer on-line to a Sigma 7 computer [24,25]. The samples were introduced into the ion source on a direct-inlet, ceramic probe and analyzed under the following conditions: resolution 10000, ionizing current 500 μA, ionizing voltage 50 eV and temp. 200°. Selected HRMS data were presented as heteroatomic plots [26]. Deviations of the HRMS fragment ion peaks from their true masses were within ± 3 millimass units.

Psilotum nudum. *P. nudum* (L.) Grisheb (*P. triquetrum* Swartz) stem (2·9 g fr. wt, 0·9 g dry wt) grown at the Botanic Gardens, University of Bristol, was trimmed into sections 15 mm long and worked up according to the method of Refs. [6] and [7]. The plant material was exhaustively extracted with MeOH (30 hr), refluxed with 3 N HCl (30 hr) and then washed with MeOH. Tissue was next digested with $ZnCl_2$–HCl (1:1·8 w/w) for *ca* 10 min and the residue poured into 5% HCl (50 ml). The cuticular membranes were removed with forceps, transferred to H_2O, dried and exhaustively extracted with toluene/MeOH (1:1). The pale brown membranes (41·3 mg) represented 4·6% dry wt of the plant stem. IR analysis (KBr disc) showed broad OH absorption (3600 cm^{-1}); bands at 2930, 2860(s) (νCH); 1735(s) (νC=O, ester); 1600(w) (νC=C, aromatic); and 1515, 1460, 1250, 1160 and 1050 cm^{-1}(w).

An aliquot of the cutin (23·5 mg) was refluxed with 5% ethanolic KOH (4 ml). The supernatant liquor and washings were acidified (dil HCl) and extracted with Et_2O (4 × 2 ml). The H_2O-washed Et_2O layer was evaporated under vacuum to an oil (6·2 mg). The lipids were methylated (CH_2N_2 in MeOH–Et_2O, 1:10) and an aliquot silylated. Direct GC–MS was carried out on the silylated total-lipid fraction. The remaining methylated lipid fraction was then subjected to

TLC on Si gel G using, Et_2O–hexane–MeOH (40:10:1). A major spot with an R_f similar to that of methyl 15-hydroxypentadecanoate (R_f 0·55) and a spot with R_f 0·10 were found. Preparative TLC using the above system afforded a major monohydroxy ester fraction and an alcohol fraction which was separated from the cutin acid methyl esters by repeated saponification and Et_2O-extraction. Both fractions were subjected to MS analysis.

The band (R_f 0·10) was identified as hexadecane-1,8,16-triol. After recrystallization of the free triol from EtOAc it had a mp of 78–79·5° (uncorr.). A micro PMR (< 1 mg sample, 30 accumulations, $CDCl_3$) at 100 MHz showed peaks at τ 6·1 (1H $\simeq$CH–OH) and τ 6·4 (4 protons, –CH_2–OH) and a multiplet at τ 8·7 (*ca* 30 protons, –CH_2–). The triol was converted to the TMSi ether, purified by preparative GLC and then analysed by micro IR (CCl_4). The IR spectrum indicated all hydroxyls to be silylated, absence of ester C=O absorption and bands at 2930 and 2860 cm^{-1} due to aliphatic C–H stretch.

Tmesipteris vieillardii. The leaf cutin from a herbarium specimen of *T. vieillardii* (Willd.) was isolated using the method of Ref. [27]. The cutin represented *ca* 9·1% of the dry wt of the leaves. IR analysis (KBr disc) of the cuticular membrane exhibited a broad OH band at 3450 cm^{-1}(w) and absorption bands at 2920(s), 1730(s), 1600(s) and 1590(w) cm^{-1}. The Et_2O-soluble hydrolysis products (2 mg) constituted *ca* 25% of the cuticular membrane. Acidification of the hydrolysate also resulted in the ppt of a brown flocculent gel which was partially soluble in BuOH, but was not further examined. A further 2 mg aliquot of membranes was treated with excess 3% aq $NaHCO_3$ [28] until no further pigment was extracted. The $NaHCO_3$ extract was not examined and the residual cutin was saponified in the normal manner, and the ether soluble lipids were analyzed.

MS. In *Psilotum* cutin hydrolysate, 8-hydroxyoctanoic and 9-hydroxynonanoic acids were identified from GC–MS of their methyl ester TMSi ethers; Methyl 8-TMSoxyoctanoate: $\dot{M}$-15, *m/e* 231 (42); 215 (7); 199 (100); 159 (6); 103 (19); 133 (16); 89 (47); 75 (85); 73 (92); 59 (50); 55 (88). Methyl 9-TMSoxynonanoate $\dot{M}$-15, *m/e* 245 (67); 229 (8); 213 (100); 103 (23); 89 (46); 75 (64); 73 (78); 55 (78); 89 (48). The mixture of non-separable Tris-TMSioxyhexadecanes derived from *Tmesipteris* cutin gave the following MS; M-15, *m/e* 475 (0·25); 400 (0·4); 391 (0·1); 385 (0·3); 377 (0·1); 363 (0·15); 359 (0·4); 345 (1·6); 331 (2·5); 317 (4·3); 303 (8·5); 289 (9); 275 (6); 261 (1·9); 247 (0·7); 233 (0·8); 221 (1·8); 149 (12); 147 (20); 171 (8·5); 185 (4); 129 (25); 137 (5); 123 (10); 109 (23); 95 (41); 85 (25); 81 (42); 75 (70); 73 (100). LRMS of 1,8,16-Tris-TMSioxyhexadecane derived from *Psilotum nudum* cutin hydrolysate: M^+, *m/e* 490 (0·1); 475 (1); 400 (1·2); 385 (1·4); 377 (1·7), 363 (1·2); 303 (80); 289 (82); 221 (2); 213 (2·5); 199 (5); 171 (1); 149 (11); 147 (25); 129 (27); 123 (15); 109 (28); 103 (38); 83 (19); 81 (29); 75 (61); 73 (100); 69 (20); 67 (26); 55 (24). HRMS of methyl 10,16-*di*-TMSioxyhexadecanoate containing 7% of the 9,16-isomer. The compounds are derived from *Cryptomeria japonica* cutin hydrolysate: *Calc. m/e* 431·301, *obs.* 431·301 ($C_{22}H_{47}O_4Si_2$); *calc.* 289·201, *obs.* 289·199 ($C_{14}H_{33}O_2Si_2$); *calc.* 275·186, *obs.* 275·185 ($C_{13}H_{31}O_2Si_2$); *calc.* 273·188, *obs.* 273·188 ($C_{14}H_{29}O_3Si$); *calc.* 259·173, *obs.* 259·171 ($C_{13}H_{27}O_3Si$). HRMS ions from 1,7,16-Tris-TMSoxyhexadecane containing 7% of the 1,8,16-isomer. This was prepared by $LiAlH_4$ reduction of the *Cryptomeria*- derived methyl dihydroxyhexadecanoates (non-separable isomers): *calc. m/e* 349·205, *obs.* 349·204 ($C_{15}H_{37}O_3Si_3$); *calc.* 317·233, *obs.* 317·231 ($C_{16}H_{35}O_2Si_2$); *calc.* 311·274, *obs.* 311·277 ($C_{19}H_{39}OSi$); *calc.* 303·217, *obs.* 303·218 ($C_{15}H_{35}O_2Si_2$); *calc.* 289·201, *obs.* 289·201 ($C_{14}H_{33}O_2Si_2$); *calc.* 275·186, *obs.* 275·186. ($C_{13}H_{31}O_2Si$).

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