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CHEMICAL DEPOLYMERIZATION STUDIES OF THE MOLECULAR ARCHITECTURE OF LIME FRUIT CUTICLE

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Key Word Index—*Citrus aurantifolia*; Citrus; Rutaceae; lime; fruit; cuticle; cutin; molecular structure; NMR; mass spectrometry; iodotrimethylsilane.

Abstract—The chemical deprotecting reagent iodotrimethylsilane has been used to achieve controlled breakdown of ester bonds in cutin, an insoluble protective biopolymer obtained from the fruit of *Citrus aurantifolia* (limes). Four soluble products that preserve essential features of the cuticular molecular architecture have been isolated chromatographically and identified by multidimensional NMR and liquid secondary-ion mass spectrometry, whereas the insoluble residue has been characterized by solid state ^{13}C NMR spectroscopy. The building blocks of the two dimers and one tetramer include the major Citrus cutin monomers 16-hydroxy-10-oxo-hexadecanoic acid and 10,16-dihydroxyhexadecanoic acid, which are joined by primary ester linkages. The CPMAS ^{13}C NMR spectra of the unreacted residue implicate a heterogeneous reaction in which esters of primary alcohols within the cross-linked cutin network are preferentially cleaved. These studies offer a direct and definitive description of molecular connectivities within the protective polyesters of fruit cuticles.

INTRODUCTION

As the support structure of leaf and fruit cuticles in higher plants, the biopolymer cutin plays a key mediating role in waterproofing and in protection of the organisms against fungal or bacterial attack. Numerous researchers have investigated cutin's molecular structure using chemical depolymerization reactions that include hydrogenolysis with $LiAlH_4$, hydrolysis with alcoholic KOH or HCl, transesterification with methanolic BF_3, and digestion with cutinases [1–3]. These degradative approaches have established that cutin is a polyester composed primarily of C_{16} hydroxylated fatty-acid building blocks, but the findings fail to provide definitive information on cross-links or other essential covalent connectivities of the cuticular support netting.

Partial enzymatic breakdown of fruit cuticles has also been reported using various lipases and cutinases [4, 5]. Although recent work using this method provided a novel pentameric fragment that retained essential cutin cross-links [6], the yield of oligomers from powdered lime cutin was a disappointing 1–11%. It was possible to obtain an 80% yield of soluble products using a more rigorous hydrolytic treatment with alcoholic KOH, and although selective retention of the esters of primary alcohols was demonstrated in the unreacted lime cutin, the soluble products obtained by this protocol were exclusively monomeric [3].

The rationale of the present study was to use a mild chemical reagent, under neutral conditions and at room temperature, in order to obtain oligomeric fragments of lime cutin. The deprotecting agent iodotrimethylsilane (TMSiI) [7, 8], which is moderately bulky and also possesses a labile Si—I bond, was chosen because it was known to cleave sterically hindered esters preferentially in organic solution [9, 10]. In the present work, lime cutin was treated with TMSiI to give a 22% yield of five soluble products. four of these materials were separated chromatographically and identified using multidimensional NMR augmented by mass spectrometry. The unreacted solid cutin residue was recovered and compared compositionally with the intact polymer using solid-state ^{13}C NMR methods.

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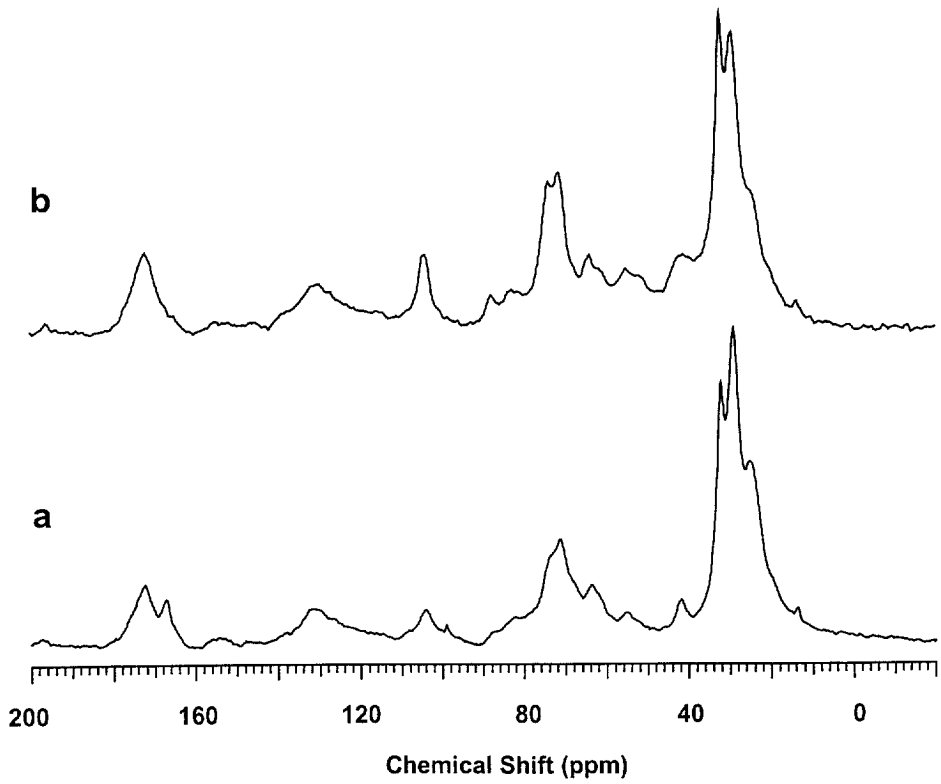


Fig. 1. ^{13}C CPMAS NMR (75.441 MHz) spectra of lime fruit cutin acquired (a) before degradative treatment; and (b) after 12 h treatment with iodotrimethylsilane. A total of 10,000 transients were averaged to obtain each spectrum, using a spectral width of 50 kHz defined after zero filling by 16K data points. Both spectra were processed with a digital line broadening of 50 Hz and plotted with the largest peak set to full scale. The chemical-shift assignments are as follows: $(CH_2)_n$, 29 ppm; $\underline{C}H_2OCOR$, 63 ppm; $\underline{C}HOCOR$ and CHOH, 72 ppm; aromatic/unsaturated, 115 and 129 ppm; $CH_2O\underline{C}OR$, 167 ppm; $CHO\underline{C}OR$, 172 ppm [13]. The resonance at 32 ppm, which may include contributions from both residual wax and cutin, also exhibits a small spinning sideband at 98 ppm.

RESULTS

Solid-state NMR analysis of unreacted residues

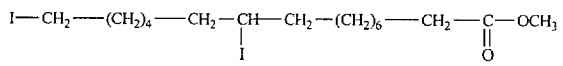
Figure 1 compares the high-resolution ^{13}C CPMAS NMR [11] data for intact lime fruit cutin and the unreacted residue remaining after treatment with TMSiI. As reported previously for alkaline hydrolysis of this biopolymer [3], the narrowing of ^{13}C signals displayed almost uniformly across the spectrum of the residue suggests that partial hydrolysis leaves behind a more homogeneous solid material [12]. Unlike the KOH reaction or the hydrolyses effected by TMSiI in homogeneous organic solution, the reaction with solid lime cutin involves cleavage of the ester linkages of primary alcohols, as evidenced by the diminution of ^{13}C resonances at 167 ppm ($CH_2O\underline{C}{=}O$). The removal of long-chain aliphatic moieties is also suggested by a decrease in their relative integrated intensity with respect to CHO groups.

Identification of soluble products by solution-state NMR and MS

Structural elucidation of the four soluble products isolated after TMSiI treatment by high-performance thin-layer chromatography (HPTLC) and column chromatography was carried out primarily using NMR spectroscopy, with important supporting information obtained from liquid secondary-ion mass spectrometry (LSIMS) [14]. As anticipated, structural analysis of these materials was facilitated by the fact that they shared the major cutin monomers, 16-hydroxy-10-oxo-hexadecanoic acid and 10,16-dihydroxyhexadecanoic acid, as common building blocks.

Compound **1** (Fig. 2), was identified as the methyl ester of 10,16-diiodohexadecanoic acid, derived in turn from 10,16-dihydroxyhexadecanoic acid, the second most abundant monomeric constituent of lime fruit cutin [3]. Its 600-MHz ^1H NMR spectrum lacked the 4.07-ppm triplet characteristic of oxymethylene protons adjacent to ester linkages (*vide infra*); its mass spectrum exhibited a molecular ion $(M+H)^+$ at m/z 523 and fragments corresponding to loss of HI and I_2, respectively.

Compound **2** was identified as a dimeric species derived from two molecules of 10,16-dihydroxyhexadecanoic acid esterified through a primary alcohol linkage (Fig. 2). In addition to diagnostic chemical shifts and integrals of the proton resonances, support for the proposed structure came from two-



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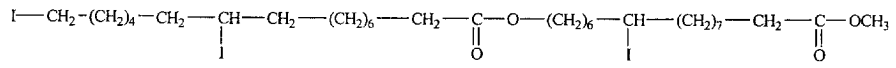

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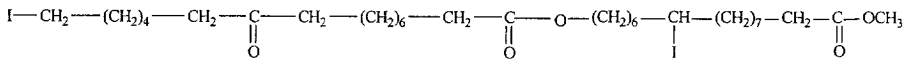

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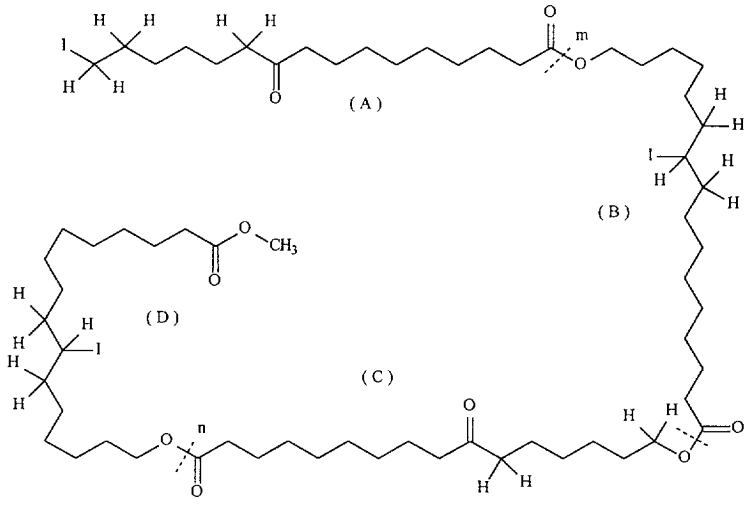

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Fig. 2. Proposed chemical structures of the isolated soluble products of lime cutin depolymerization with TMSiI. For the tetramer **4**, the monomeric units are designated (A)–(D) and the putative MS cleavage sites are designated *l*–*n*.

dimensional NMR experiments that correlate chemically bonded ^{1}H and ^{13}C nuclear spins. Within a given monomer unit, ^{1}H-^{1}H total correlated spectroscopy (TOCSY) [15] was used to verify through-bond connections; connectivities that span up to five bonds were observed in compound **2**, as has been reported previously for saturated aliphatic chains that adopt a zig-zag arrangement [16]. ^{1}H-^{13}C gradient-assisted heteronuclear multiple quantum correlation (HMQC) [17] was used to detect directly-bonded carbon-proton pairs. In addition, the presence of ester carbonyl groups within each monomer unit was established by ^{1}H-^{13}C gradient-assisted heteronuclear multiple-bond (long-range) correlations (HMBC) [18] to the near-neighbor protons at (166.8, 2.30 ppm) and (167.0, 2.31 ppm), respectively.

Evidence for the covalent ester linkages between the two monomers was also obtained through HMBC experiments. In particular, we observed a multiple-bond correlation (166.8, 4.07 ppm) between the central carbonyl carbon and protons bound to the oxymethylene group. Finally, the proposed dimer structure was supported by LSIMS, which gave a molecular ion $(M+H)^{+}$ with m/z 903 ($C_{33}H_{61}O_4I_3$) as well as ions attributable to the loss of one and two HI molecules, respectively. The two identical constituents of depolymerization product **2** have been reported previously as the second most abundant monomer of lime fruit cutin [3].

In similar fashion, one- and two-dimensional NMR evidence was combined with the results of LSIMS to identify the dimer **3** (Fig. 2). Many of the chemical shift and connectivity trends resembled those described for **2**, since these compounds share the 10,16-dihydroxyhexadecanoic acid monomer unit. However, the replacement of compound **2**'s second monomer unit by 16-hydroxy-10-oxo-hexadecanoic acid, i.e., the appearance of a $CH_2C(O)CH_2$ group, was supported by several lines of evidence: (a) integrals showing 4 protons (2.4 ppm) in place of one methine (4.1 ppm) and two methylenes (1.65, 1.85) adjacent to a CHI unit; (b) protons (2.4 ppm) linked

by HMQC to a carbon resonance at 43 ppm; (c) protons (2.4 ppm) linked by HMBC to a keto carbon resonance at 197.5 ppm.

The TOCSY connectivities to methylene protons that flank the keto carbon were notable, since they included a 4-bond coupling to protons at 1.35 ppm as well as a weak 6-bond coupling to protons at 1.85 ppm. This pattern indicated that the keto group should be placed at the 10-position; that is, it allowed us to identify 16-hydroxy-10-oxo-hexadecanoic acid, the most abundant monomer in lime fruit cutin [3], as a constituent of dimer **3**. LSIMS data were in full accord with the proposed structure, yielding the anticipated molecular ion $(M+H)^+$ with *m/z* 791 ($C_{33}H_{60}O_5I$) and $(MH-HI)^+$ with *m/z* 663.

Structural elucidation of the tetramer **4** (Fig. 2) relied primarily on NMR data, since the molecular ion $(M+H)^+$ predicted at *m/z* 1581 was not detected by LSIMS or other soft-ionization mass spectral methods. As compared with dimer **3**, no new resonances were evident in the one-dimensional ^1H NMR spectrum, suggesting first of all that compound **4** has the same 10,16-dihydroxyhexadecanoic acid and 16-hydroxy-10-oxo-hexadecanoic acid building blocks (or possibly their close positional isomers). Evidence for a tetrameric structure came from a collection of integrated peak intensities: (a) methoxy protons at 3.69 ppm were taken as 3H; (b) the CHI protons (4.1 ppm, 2H) were indicative of two dihydroxy C_{16} monomeric units; (c) the CH_2's flanking a keto group (2.4 ppm, 8H) were consistent with two hydroxyoxo C_{16} units; (d) the ester-bonded CH_2's (2.3 ppm, 8H) indicated four ester groups (3 internal, 1 terminal methyl ester); (e) the bulk-methylene protons (1.35–1.6 ppm, 82H) followed the predictions corresponding to two units each of 10,16-dihydroxyhexadecanoic acid (21H) and 16-hydroxy-10-oxo-hexadecanoic acid (20H).

Additional support for the proposed structure came from TOCSY, HMQC, and HMBC experiments, which reproduced the spectral features discussed above for dimers **2** and **3**. The observation of multiple-bond TOCSY connectivities reinforced the hypothesis of 10,16-dihydroxyhexadecanoic acid and 16-hydroxy-10-oxo-hexadecanoic acid monomer units, since both have long methylene "runs" that may adopt a zig-zag conformation and facilitate spin communication to the CHI and CH_2I protons, respectively. The two overlapping triplets at 2.4 ppm indicated a chemical-shift inequivalence between the two 16-hydroxy-10-oxo-hexadecanoic acid building blocks, attributable to an ordered conformation for tetramer **4** or to an environmental distinction between terminal and internal locations of these monomer units. All of these considerations taken together suggested the tentative structure shown in Fig. 2.

Although mass spectrometry yielded no molecular ion for compound **4**, a sufficient number of dimeric fragments were found in the spectrum to confirm the sequential arrangement of monomers proposed for this lime cutin fragment (Fig. 2). For instance, it was possible to identify strong peaks corresponding to A+B (*m/z* 759, $C_{32}H_{57}O_4I_2$), B+C−H (*m/z* 647, $C_{32}H_{57}O_5I$), and C+D (*m/z* 679, $C_{33}H_{60}O_6I$). A full analysis of the LSIMS fragmentation patterns for the cutin oligomers will be presented elsewhere.

Finally, preliminary structural data were obtained from NMR spectra of the band retained at the origin in HPTLC. This material, which is likely to be a high-molecular-weight mixture of cutin oligomers, exhibited ^1H NMR spectra very similar to tetramer **4**.

DISCUSSION

The studies presented herein provide a direct and definitive description of molecular connectivities within the protective polyesters of fruit cuticles. Using a mild chemical reagent to generate oligomeric fragments of lime cutin, it has been possible to augment existing compositional information derived from spectroscopic examination of the monomeric constituents [3] and the intact solid biopolymer [13]. The constituents of these oligomers correspond to the most abundant monomers derived from alkaline or acidic depolymerization [1–3]. Both the current chemical degradation studies and our recent enzymatic depolymerization results [6] support a molecular architecture in which esters of primary and secondary alcohols form the predominant linkages between fatty-acid units of the cutin biopolymer. These findings also contain the elements of a model for the design of synthetic polymer blends that could be used in water-proofing applications.

Several aspects of the chemistry of cutin depolymerization are also noteworthy. Whereas CPMAS ^{13}C NMR indicates that esters of primary alcohols are removed preferentially upon treatment of powdered lime cutin with TMSiI, no esters of secondary alcohols survive among the soluble oligomeric products. Thus the well-known efficiency of this reagent in promoting the cleavage of sterically hindered esters [9, 10] may be reestablished once the soluble cutin fragments have been broken off from the solid starting material. This hypothesis regarding TMSiI contrasts with the strict selectivity of porcine pancreatic lipase for hydrolysis of esters of primary alcohols, producing a soluble pentamer in which only secondary ester linkages are retained.

EXPERIMENTAL

Isolation of lime cutin

Cutin was isolated from the skin of limes (*Citrus aurantifolia*) by published procedures [19]. In brief, the procedure involved three steps: (1) peeling and separation of the cuticle by treatment with *Aspergillus niger* pectinase; (2) enzymatic degradation of carbohydrates with cellulase, pectinase, and hemicellulase; (3) exhaustive dewaxing by Soxhlet extraction with

methanol, methylene chloride, and tetrahydrofuran. All chemicals were obtained from Sigma or Aldrich chemical companies. Typically, 250 limes yielded 4.5 g of dry cutin.

Chemical depolymerization

Powdered cutin was produced using a freezer mill cooled with liquid nitrogen (Spex Industries, Edison, NJ). A 500 mg sample of the biopolyester was suspended in 25 ml of dry CCl_4 and stirred for 30 min. Then 5 g of TMSiI were added to form a mixture that was stirred at 22°C for 12 h. The progress of the reaction was monitored by the appearance of two sharp IR absorption bands at 1700 cm^{-1} (acid carbonyl) and 1738 cm^{-1} (ester carbonyl) [20] (Nicolet Magna 550 spectrometer). The mixture was filtered, after which the filtrate was washed with water and then 5% aqueous sodium thiosulfite to remove excess iodine. The resulting product mixture was dried over anhydrous sodium sulfate for 1 h and evaporated to dryness under reduced pressure. 250 mg of a solid residue was recovered and reserved for solid-state NMR analysis. Workup of the filtrate afforded 110 mg (22% yield) of a light brown material. Finally, treatment of this material with 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich) in dry ether gave a diazomethane reaction to yield an ester product mixture with IR absorption at 1738 cm^{-1}.

Isolation of soluble products

High-performance preparative thin-layer chromatography (HPTLC) was conducted using silica gel 60 plates (10 × 20 cm, 0.5 mm thickness) from EM Science, 3:2 methylene chloride: *n*-hexane as the customary developing solvent, and iodine for visualization. The band at the origin contained 10 mg of an ether-insoluble material. The remaining four bands were separated, eluted with 2:1 chloroform:methanol, and washed through a silica gel column with *n*-hexane. Four colorless viscous compounds were isolated from HPTLC bands with R_f values of 0.82 (**1**, 4.2 mg), 0.64 (**2**, 4.9 mg), 0.50 (**3**, 5.8 mg) and 0.37 (**4**, 5.7 mg), respectively.

Solution-state NMR spectroscopy

^{1}H nuclear magnetic resonance (NMR) spectra were acquired on a Varian Unity*plus*-600 spectrometer operating at a proton frequency of 599.95 MHz. Samples were dissolved in $CDCl_3$ containing 1% tetramethylsilane (Wilmad Glass Company, Buena, NJ) to provide a field-frequency lock and an internal chemical shift standard, respectively. One-dimensional spectra were typically acquired with 1500 transients, using 23,000 time-domain points and a spectral width of 6000 Hz. Two-dimensional ^{1}H-^{1}H experiments (TOCSY) [15] were used to delineate chemically bonded proton networks within each monomer unit. In addition, bonded proton-carbon pairs and long-range proton-carbon bonded interactions were identified with two-dimensional ^{1}H-^{13}C experiments (HMQC [21] and HMBC [22], respectively).

The "clean" TOCSY experiments [22] were conducted with an effective spin-lock field of 7 kHz and a mixing time of 70 ms to correlate all protons within a bonded network. For the HMQC and HMBC experiments, pulsed-field gradient methods [17, 18] were used to minimize spectral artifacts, and the GARP method [24] was employed for ^{13}C decoupling. Typical acquisition conditions included spectral widths of 6.5 kHz for ^{1}H and 30 kHz for ^{13}C, respectively, polarization-transfer times corresponding to scalar couplings $^1J_{CH} = 140$ Hz and $^3J_{CH} = 9$ Hz, and a relaxation delay of 1.5 s. The data were collected as arrays of 2K × 256 points, which after zero filling resulted in a 2K × 1K data matrix.

Monomer **1**. ^{1}H NMR: δ 1.2–1.7 (*br s*, $(CH_2)_n$, 20H), δ 1.8, (*m*, $C\underline{H}_2CHI$ and $C\underline{H}_2CH_2I$, 4H, distinguished by TOCSY crosspeaks to resonances at 4.1 and 3.2 ppm, respectively), δ 2.3 (*t*, CH_2COO, 2H), δ 3.2 (*t*, $C\underline{H}_2I$, 2H), δ 3.69 (*s*, $CH_2COOC\underline{H}_3$, 3H), δ 4.1 (*m*, CHI, 1H). Dimer **2**: ^{1}H NMR: δ 1.35 (*br s*, 22H), δ 1.5 (*br s*, 10H), δ 1.65 (*br s*, 10H), δ 1.85 (*m*, 6H), δ 2.3 (2*t*, 4H), δ 3.2 (*t*, 2H), δ 3.67 (*s*, 3H), δ 4.07 (*t*, $CH_2COOC\underline{H}_2$, 2H) and δ 4.1 (*m*, 2H). Dimer **3**: Viscous; ^{1}H NMR: δ 1.35 (*br s*, 22H), δ 1.50–1.65 (*br s*, 18H), δ 1.85 (*m*, 4H), δ 2.3 (2*t*, 4H), δ 2.4 (2*t*, 4H), δ 3.2 (*t*, 2H), δ 3.67 (*s*, 3H), δ 4.07 (*t*, 2H), and δ 4.1 (*m*, 1H). Tetramer **4**: Pale yellow oily; ^{1}H NMR: δ 1.35–1.6 (*br s*, 82H), δ 1.85 (*m*, 6H), δ 2.3 (*q*?, 8H), δ 2.4 (2*t*, 8H), δ 3.2 (*q*?, 2H), δ 3.67 (*s*, 3H), δ 4.07 (*q*?, 6H) and δ 4.1 (*m*, 2H).

Solid-state NMR spectroscopy

The NMR spectra were acquired on a Varian Unity*plus*-300 widebore spectrometer (Varian NMR Instruments, Palo Alto, CA) equipped for high-resolution solid-state NMR and operating at a ^{13}C resonance frequency of 75.441 MHz. A Varian 7-mm cross polarization/magic-angle spinning (CPMAS) probe operating at a temperature of 23 ± 1° was used to acquire the data; typical conditions included MAS of 5000 ± 5 Hz, 2.5-ms Hartmann-Hahn matching at a radiofrequency strength of 40 kHz, and 40-kHz ^{1}H decoupling during signal acquisition. The ^{13}C spectral width was 50 kHz defined by 16K points; a recycle delay of 5 s was inserted between successive transients to permit spin relaxation. Chemical shifts are quoted with respect to TMS using the bulk-methylene resonance (29 ppm) of solid lime cutin [13] as a secondary substitution reference. Integrated intensities for each of the major functional groups were estimated by cutting and weighing of portions of the spectral traces.

Mass spectrometry

Liquid secondary ionization mass spectrometry (LSIMS) was carried out using a VG ZAB-T high-

resolution mass spectrometer (VG Analytical, Manchester, England). A cesium ion gun operating at 30 kV was used as the ionizing source, and an accelerating potential of 8 kV allowed observation of ions within the range 50–2000 a.m.u. A 3:1 dithiothreitol/dithioerythritol "magic bullet" matrix was used to prepare the samples. Data were acquired and processed in the continuum mode, with averaging of 100 spectra carried out using an Opus data system.

Monomer **1**. $(M+H)^+$ *m/z* 523; $(MH-HI)^+$ *m/z* 395; $(MH-I_2)^+$, cyclized to seven-membered ring, *m/z* 269; CH_2-C-$(CH_2)_6$-CH_2-$COOCH_3^+$ *m/z* 171. Dimer **2**: $(M+H)^+$ *m/z* 903; $(MH-HI)^+$ *m/z* 775; $(MH-2HI)^+$ *m/z* 647; $(MH-I_2)^+$, cyclized to seven-membered ring *m/z* 649. Dimer **3**: $(M+H)^+$ *m/z* 791; $(MH-HI)^+$ *m/z* 663; $(MH-2HI)^+$ *m/z* 535. Tetramer **4**: $(A+B)^+$ *m/z* 759; $(B+C-H)^+$ *m/z* 647; $(B+C-H-HI)^+$ *m/z* 519; $(C+D)^+$ *m/z* 679; $(C+D-HI)^+$ *m/z* 551.

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