Biosynthesis, Molecular Structure, and Domain Architecture of Potato Suberin: A ^{13}C NMR Study Using Isotopically Labeled Precursors

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Although suberin in potato wound periderm is known to be a polyester containing long-chain fatty acids and phenolics embedded within the cell wall, many aspects of its molecular structure and polymer−polymer connectivities remain elusive. The present work combines biosynthetic incorporation of site-specifically ^{13}C-enriched acetates and phenylalanines with one- and two-dimensional solid-state ^{13}C NMR spectroscopic methods to monitor the developing suberin polymer. Exogenous acetate is found to be incorporated preferentially at the carboxyl end of the aliphatic carbon chains, suggesting addition during the later elongation steps of fatty acid synthesis. Carboxyl-labeled phenylalanine precursors provide evidence for the concurrent development of phenolic esters and of monolignols typical of lignin. Experiments with ring-labeled phenylalanine precursors demonstrate a predominance of sinapyl and guaiacyl structures among suberin's phenolic moieties. Finally, the analysis of spin-exchange (solid-state NOESY) NMR experiments in ring-labeled suberin indicates distances of no more than 0.5 nm between pairs of phenolic and oxymethine carbons, which are attributed to the aromatic−aliphatic polyester and the cell wall polysaccharide matrix, respectively. These results offer direct and detailed molecular information regarding the insoluble intermediates of suberin biosynthesis, indicate probable covalent linkages between moieties of its polyester and polysaccharide domains, and yield a clearer overall picture of this agriculturally important protective material.

Keywords: *Suberin; cell wall; potato; wound healing; Solanum tuberosum; biosynthesis; polymer; polyester; polysaccharide; ^{13}C NMR; solid-state NMR; CPMAS; spin-exchange; spin diffusion; NOESY*

INTRODUCTION

Suberized potato tissue, a polymeric plant material isolated readily from wound periderm, has long been a model system for studying the molecular structure and protective functions of suberin. Its chemical constituents include long-chain fatty acids as the aliphatic component (Kolattukudy, 1980, 1984) and phenolic derivatives as the aromatic component (Cottle and Kolattukudy, 1982; Bernards and Lewis, 1992; Bernards et al., 1995). The aliphatics and aromatics are believed to be linked by ester bonds (Kolattukudy, 1980), a hypothesis consistent with the analysis of suberin extracts (Bernards and Lewis, 1992). Even after exhaustive enzymatic removal of carbohydrates and extraction with organic solvents, the aliphatic−aromatic suberin polymer remains insoluble and inseparable from the cell wall polysaccharides. Thus, suberized potato may be a tightly associated plant polymer blend or a copolymer in which different domains are linked covalently to each other (Bernards and Lewis, 1998; Yan and Stark, 1998). Nonetheless, the polymerization steps of its biosynthesis are largely unknown, and the nature of the covalent connectivities within suberin and to the cell wall polysaccharides has remained quite speculative. These limitations hamper efforts to understand or improve upon the ability of the biopolymer to protect cell wall tissues.

Historically, two general approaches have been taken to the study of suberin's molecular structure. First, depolymerization or extraction methods may be used along with nuclear magnetic resonance (NMR) and mass spectrometry to identify monomeric and oligomeric fragments and then conceptually reconstruct the original polymer (Kolattukudy and Agrawal, 1974; Cottle and Kolattukudy, 1982; Bernards and Lewis, 1992). Alternatively, it is possible to identify structural types directly within intact suberin using solid-state NMR (Stark et al., 1989, 1994; Stark and Garbow, 1992). The information content of the former approach may be compromised by either incomplete depolymerization (biased representation of the biopolymer) or exhaustive breakdown (destruction of connectivities between the monomeric units); multistep chemical procedures are usually also required. The latter strategy provides information that is more directly applicable to the native plant membrane, but the structural complexity of suberized plant tissue can make it challenging to deduce detailed molecular information unless the polyester is labeled with spectroscopically sensitive isotopes (Bernards et al., 1995).

In the present work, both aliphatic and aromatic ^{13}C-labeled precursors have been used along with solid-state NMR methods to obtain new molecular-level information regarding the biosynthesis and domain architecture of potato suberin. The biosynthetic fates of acetate and

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phenylalanine precursors have been examined qualitatively and quantitatively for the intact plant tissue, making careful comparisons with unlabeled materials and gaining additional information from spectral editing experiments. In addition, two-dimensional solid-state spin-exchange ^{13}C NMR has been used to critically evaluate the covalent connectivity of suberin aromatics to carbons of the phenylpropanoid side chain or the cell wall polysaccharides. These findings augment our molecular picture of the mechanism by which suberin controls water diffusion and protects cell wall polysaccharides from pathogenic attack.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were purchased from Cambridge Isotope Laboratories (Andover, MA): [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]sodium acetates; [1-^{13}C]- and [*ring*-$^{13}C_6$]-L-phenylalanine. All materials were enriched to 99% or better with stable isotopes. Tissue culture water (Sigma Chemical, Aurora, OH) was used to dissolve the ^{13}C-enriched compounds and moisten the suberizing wound periderm in each experiment. The enzymes *Aspergillus niger* cellulase (EC 3.2.1.4) and *A. niger* pectinase (EC 3.2.1.15) were purchased from ICN Biomedicals (Aurora, OH) and Sigma Chemical, respectively. Other laboratory chemicals were of reagent grade or better.

Preparation of Enriched Potato Suberin. Suberization of wounded potatoes (*Solanum tuberosum* L. cv. Russet Burbank) and suberin isolation followed published procedures (Pacchiano et al., 1993; Bernards et al., 1995; Yan and Stark, 1998). In separate experiments, each batch of sterilized potatoes was cut into 5 × 20 × 30-mm^3 disks and then soaked in 100 mM solutions of the ^{13}C-enriched precursors for 20 min before incubation in a dark aerated chamber regulated at 25 °C. To minimize any effects of changing concentration as a particular chemical solution was applied to successive tuber samples, the slices were arranged in a rectangular grid so that soaking proceeded across the rows and harvesting proceeded down the columns. This precaution was especially important for time course studies in which the tubers were harvested after varying incubation times (1, 3, 5, 7, and 10 days, respectively). The post suberization isolation procedures were as follows: blade peeling of the periderm layer from each potato slice; thorough stirring with distilled water to remove soluble starch and residual precursors; treatment with cellulase and pectinase to remove cellulose and pectin, respectively; Soxhlet extraction of the pea-sized flakes of periderm with methylene chloride−methanol (1:1 v/v) to remove waxes; extraction with dioxane−H_2O (96:4 v/v) to remove residual glucose or soluble saccharides. In control experiments, periderm soaked in solutions of natural-abundance sodium acetate precursors was found to yield very similar solid-state ^{13}C NMR spectra to those published previously, and periderm soaked in water alone was verified to be comparable spectroscopically to the suberized tissue generated using 100 mM [^{12}C]acetate solutions (Stark et al., 1994; Bernards et al., 1995; Yan and Stark, 1998).

NMR Spectroscopy. Dry suberin samples were analyzed using standard cross-polarization magic-angle-spinning (CPMAS) ^{13}C experiments carried out on a Varian Instruments Unity*plus* 300 widebore spectrometer (Palo Alto, CA) equipped for solid-state NMR. The operating frequency was 75.443 MHz for ^{13}C, with a typical acquisition time of 30 ms, delay time of 2 s between successive transients, and CP contact time of 1.5 ms. A 5-mm supersonic MAS probe from Doty Scientific (Columbia, SC) was used at ambient temperature with a rotor spinning speed of 9.000 ± 0.005 kHz and a ^{1}H decoupling strength of 60 kHz.

The selective suppression of signals from protonated carbons was achieved using a dipolar-dephasing scheme (Opella and Frey, 1979), in which a delay of 64 μs was inserted before the signal acquisition time. For studies of possible linkages between polymeric domains, a two-dimensional (2D) ^{13}C−^{13}C spin-exchange experiment was modified so that the initial ^{13}C magnetization was built up by cross-polarization instead of using a simple 90° pulse (Bardet et al., 1997). This sequence is essentially a 2D nuclear Overhauser experiment (Jeener et al., 1979) in which cross-talk is allowed between dipolar-coupled ^{1}H and ^{13}C nuclei during mixing times of 0.01−2 s, with consequent changes in off-diagonal ^{13}C cross-peak intensity. Spin diffusion among the carbons is driven by the protons; no ^{1}H decoupling is applied during the mixing period. The 2D spectra were obtained by collecting 64 transients of 960 points each for each of 256 time increments, applying a line broadening of 200 Hz, and zero filling to 1024 data points in both dimensions before the final 2D Fourier transformation and data symmetrization. Spectral analyses were performed using the Varian VNMR software package, with ^{13}C chemical shifts referenced to tetramethylsilane (TMS) (Pacchiano et al., 1993) or the major polysaccharide peak at 72 ppm. Chemical-shift predictions for aromatic acids were made using database software from Advanced Chemistry Development (Toronto, Canada).

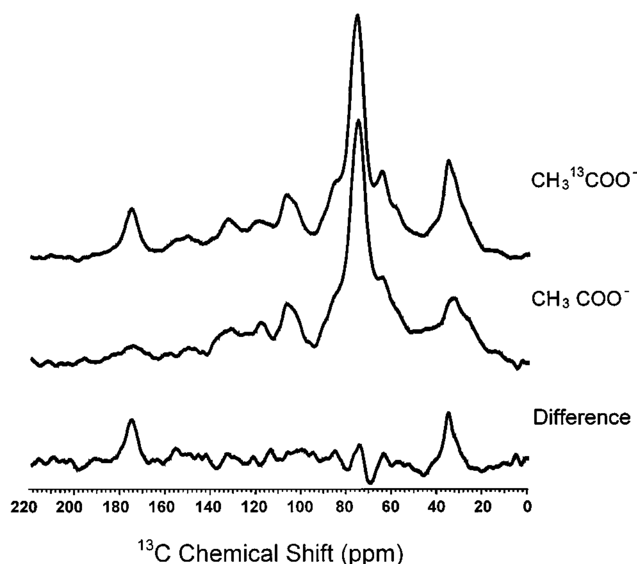


Figure 1. Effects of a [1-^{13}C]sodium acetate precursor on the CPMAS ^{13}C NMR spectra of potato suberin: (top) ^{13}C-Labeled sample; (middle) natural-abundance (^{12}C) control sample; (bottom) Difference spectrum. Both samples were incubated for 7 days after wounding. The peak areas in each spectrum reflect the numbers of the various carbon types, since CP buildup is complete within 0.8−1.1 ms and intensity losses from proton relaxation in the rotating frame are closely comparable (Stark and Garbow, 1992).

RESULTS AND DISCUSSION

Incorporation of Acetate Precursors into Long-Chain Fatty Acids. It is well-established from chemical depolymerization studies that the aliphatic chains in suberin consist of predominantly C_{18} and C_{16} homologues (Kolattukudy and Dean, 1974; Kolattukudy and Agrawal, 1974). The biosynthetic pathways that produce these aliphatic monomers have been proposed by analogy with reactions that take place between acetyl-CoA and malonyl-CoA in the presence of fatty acid synthetases (Kolattukudy, 1984). To determine the biosynthetic fate of aliphatic constituents incorporated into the developing polymer during wound healing, the present study used site-specific ^{13}C-enriched precursors and subsequent examination of the suberized tissue by CPMAS ^{13}C NMR spectroscopy. Figures 1 and 2 show the spectra of suberized potato cell walls exposed to singly and doubly labeled acetate precursors, with the

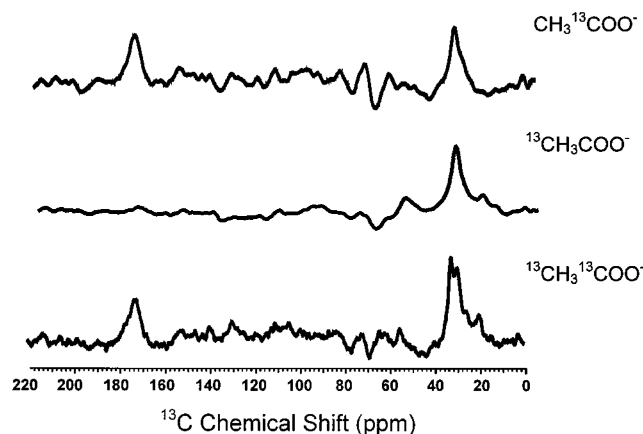


Figure 2. Difference NMR spectra resulting from ^{13}C labeling of potato suberin with three acetate precursors: (top) [1-^{13}C]-acetate; (middle) [2-^{13}C]acetate; (bottom) [1,2-$^{13}C_2$]acetate. Each sample was incubated for 7 days after wounding. Artificial line broadening of 100 Hz was applied to process the top and middle spectra, but 60 Hz was applied to the bottom spectrum in order to highlight the presence of two resonances in the 30−33 ppm region. The peak areas reflect the numbers of the various carbon types, as explained in the caption to Figure 1.

sites of ^{13}C enrichment highlighted by comparison to analogous data obtained from experiments with ^{12}C-based precursors.

Compared with the control sample, [1-^{13}C]acetate-labeled suberin exhibits two new ^{13}C NMR signals at 32 and 173 ppm (Figure 1). The carboxyl peak at 173 ppm is expected, because the C_1 of acetate can be incorporated into the fatty acid carboxyl groups of suberin. Moreover, HCO_3^- carboxylation catalyzed by acetyl-CoA carboxylase may convert acetate into malonyl-CoA, which provides the two-carbon building blocks used in synthesis of long-chain fatty acids (Kolattukudy, 1984; Stryer, 1988). Thus, the $^{13}C_1$ of acetate could be incorporated biosynthetically into each of the odd-numbered carbons of C_{16} or C_{18} fatty acids, building up the NMR signal intensity of the 32-ppm bulk-methylene resonance. In addition, the formation of major suberin monomers such as octadec-9-ene-1,18-dioic acid and 18-hydroxyoctadec-9-enoic acid (Kolattukudy and Dean, 1974; Holloway, 1983) would yield a measurable resonance at ∼130 ppm, corresponding to isotopic labeling at the C_9 position. However, the difference spectrum of Figure 1 shows that the 173 ppm signal has an integrated intensity about 60% as large as the 32 ppm peak, indicating that the exogenously administered acetate is incorporated disproportionately at the carboxyl ends of the final ω-hydroxyalkanoic and α,ω-dioic acids.

This labeling pattern may be rationalized if exogenous [1-^{13}C]-labeled acetate is involved only in elongation of the fatty acid chains rather than de novo biosynthesis. Available malonyl-CoA or short-chain acids may be used to synthesize palmitoleic (16:1) or oleic (18:1) fatty acids in the plastids. By contrast, the enriched acetate precursor will be present in the cytoplasm, where it may be incorporated (as acetyl-CoA) during chain elongation occurring on the endoplasmic reticulum. Since the exogenous acetate adds to a partially elongated fatty acid fragment, it will not label all possible aliphatic carbon positions evenly under the experimental conditions typically used in our experiments. Moreover, analysis of the ^{13}C-enriched depolymerization products will give an inaccurate estimate of the fatty acid composition in suberin.

The [2-^{13}C]acetate-labeled suberin displays just one significant new ^{13}C NMR peak at ∼30 ppm (Figure 2, middle). On the basis of results described above for [1-^{13}C]acetate, we expect labeling at the even-numbered carbons, but principally at those sites close to the terminal carboxyl group. Although small chemical shift differences between, say, C_2, C_4, and C_6 would be indistinguishable within the broad envelope of the methylene-carbon peak, labeling at C_{10} would give rise to an unsaturated carbon resonance (∼130 ppm) as described above. Labeling at several fatty acid sites was suggested by preliminary incorporation experiments conducted with a different laboratory protocol (Stark et al., 1994), but only labeling of the chain methylenes is evidenced by the current CPMAS ^{13}C NMR data. These results support the hypothesis of ^{13}C incorporation into partially synthesized fatty acids.

The solid-state NMR data for the [1,2-$^{13}C_2$]acetate-labeled suberin (Figure 2, bottom) confirm the observations made with the singly labeled precursors. As expected, the spectrum includes all the enhanced peaks present in the top and middle traces of Figure 2. Moreover, the region of the ^{13}C NMR spectrum near 30 ppm shows two resolved peaks, which are attributable to methylene groups at different fatty acid chain locations.

Incorporation of Carboxyl-Labeled Phenylalanine into Hydroxycinnamic Acids and Monolignols. Structural information on the aromatic domain of suberin has been obtained previously by several contrasting strategies: depolymerization using alkaline nitrobenzene oxidation (Cottle and Kolattukudy, 1982); thioacidolysis to degrade alkyl−aryl ethers (Borg-Olivier and Monties, 1989); solvent extraction from the wound-healing tissue (Bernards and Lewis, 1992); and NMR examination of purified potato wound periderm tissue formed in the presence of ^{13}C-enriched phenylalanine precursors (Bernards et al., 1995). Although the occurrence of *p*-hydroxycinnamic acid-derived chemical structures is indicated in each of these studies, the question of whether "lignin-like" monolignols (Cottle and Kolattukudy, 1982) or phenolic esters (Bernards et al., 1995) predominate in the suberin polymer has been more controversial. To clarify this important structural issue, we reexamined the incorporation of [1-^{13}C]phenylalanine into potato suberin using NMR spectroscopy and a protocol of varying incubation times.

Figure 3 shows the CPMAS ^{13}C NMR spectra of suberin after 7 days of incubation and completion of all purification treatments. The difference spectrum (bottom) reveals two ^{13}C-labeled peaks of almost equal integrated intensity: carboxyl groups resonating at 173 ppm and hydroxymethyl structures giving rise to a peak at 63 ppm. This finding suggests that a significant number of lignin-like monolignol structures (**1**) exist within the suberin in addition to the hydroxycinnamic acid (**2**) or ester functionalities found to be predominant in prior studies (Bernards et al., 1995) (see Scheme 1). To check that both labeled moieties were generated during suberization rather than during the purification process, we confirmed that the intensity relationship between the 63 and 173 ppm signals was reasonably constant throughout the different enzymatic and extractive treatments (Figure 4). To check whether the proportion of monolignol present was associated with

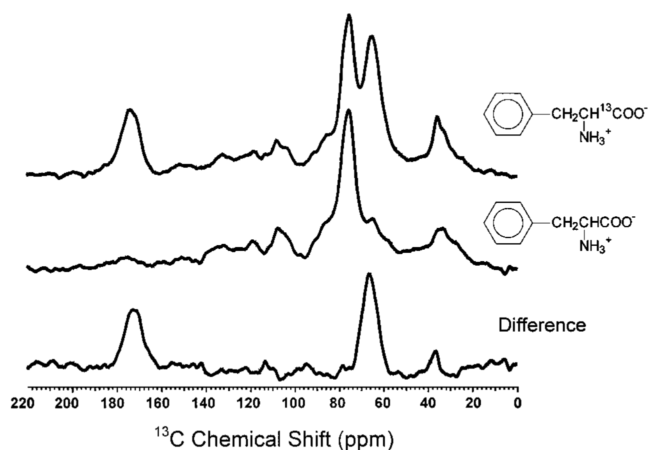
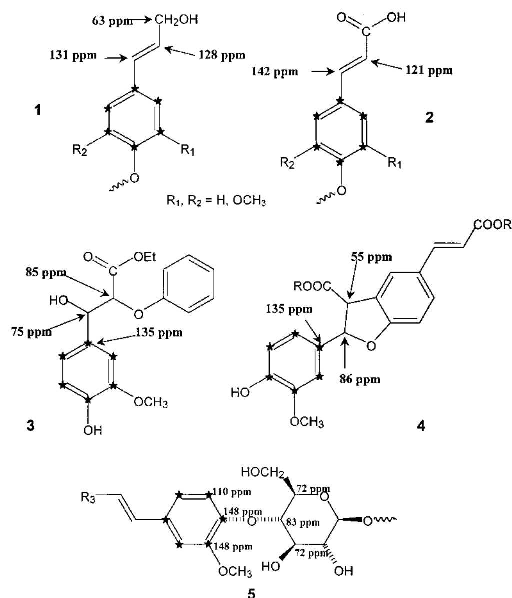


Figure 3. Effects of a [1-^{13}C]-phenylalanine precursor on the CPMAS ^{13}C NMR spectra of potato suberin: (top) ^{13}C-labeled sample; (middle) natural-abundance (^{12}C) control sample; (bottom) difference spectrum. Both samples were incubated for 7 days after wounding. Resonances are assigned to CH_2OH of monolignols (63 ppm) and COO of hydroxycinnamic acid (173 ppm), with chemical structures **1** and **2** shown in Scheme 1.

Scheme 1



differences in the way the precursors were introduced, we also administered the phenylalanine solutions onto the tissue surface directly (Bernards et al., 1995), instead of soaking the cut potato with precursor solutions for 20 min. The ^{13}C NMR spectra collected after 7 days of suberization remained unchanged, and the monolignol-to-hydroxycinnamic acid ratio was maintained to within 10%.

To follow the formation of the aromatic domain more closely, a time course study was conducted using a [1-^{13}C]phenylalanine (Phe) precursor and wound-healing periods of 0−10 days. Figure 4 summarizes the variation with time of ^{13}C NMR signal intensities at 173 and 63 ppm, showing that the buildup of *both* acid and monolignol structures within suberin begins after 1 day, reaches a maximum after ~5 days, and then stabilizes. As noted above for the 7-day incubation, the relative signal intensities of the two molecular entities are reasonably consistent at every stage of postsuberization purification. In addition to confirming the quantitative

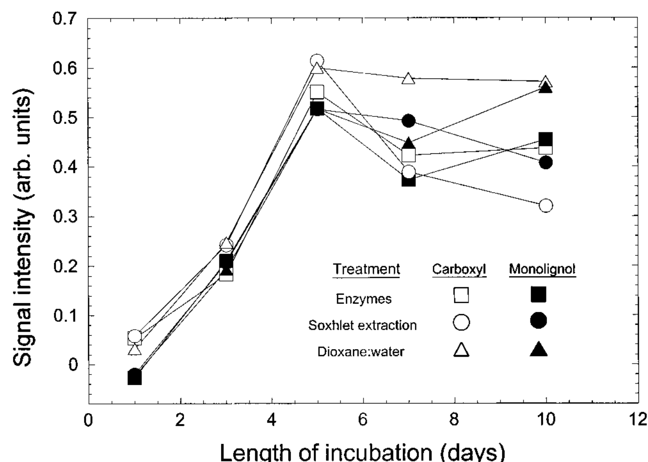


Figure 4. Time course study of incorporation for a [1-^{13}C]-phenylalanine precursor into the aromatic domain of potato wound periderm, monitored through CPMAS ^{13}C NMR spectra obtained after 0−10 days. Integrated signal intensities are plotted for carboxyl groups (173 ppm, open symbols) and monolignols (63 ppm, closed symbols) after different stages of the postincubation treatment: Enzyme treatments, □ and ■; organic solvent extraction, ○ and ●; dioxane-H_2O extraction, △ and ▲). The monolignol-to-hydroxycinnamic acid ratio varied by 10% in duplicate preparations.

significance of both acid and monolignol structures, the time course study reveals significant temporal relationships involving the formation of various molecular structures. First, monolignols are produced concurrently with their acid counterparts. Second, the time frame for development of the two labeled moieties derived from aromatic precursors matches the incorporation of [^{14}C]-acetate into potato suberin aliphatics (Dean and Kolattukudy, 1977) and coincides with the development of diffusion resistance in the wound-healing tissue (Kolattukudy and Dean, 1974). The relative amounts of hydroxycinnamic acid and monolignol products appear to depend on details of the suberization protocol followed in different laboratories (Bernards et al., 1995), including the concentration of precursors compared with endogenous metabolites, pH and oxygen concentration at the periderm surface, timing and duration of incubation, and consistency of subsequent purification procedures. These variations in biosynthetic outcomes also offer the potential of modulating the formation of essential protective structures by proper choice of experimental conditions.

Incorporation of Ring-Labeled Phenylalanine into Guaiacyl and Sinapyl Acids. Turning to the chemical nature of the phenolic suberin moieties, appropriate ^{13}C-labeled precursors were used to distinguish *p*-coumaroyl, guaiacyl, and sinapyl aromatic ring structures (Davin and Lewis, 1992). These aromatic ring structures have been identified and quantified previously in suberin monomers from root tissues (Zeier and Schreiber, 1997; Zeier et al., 1999), but the partial nature of the degradative treatments prompted us to design an alternative in situ protocol for wound periderm. By incorporating [*ring*-$^{13}C_6$]phenylalanine into potato wound periderm, it was possible to use ^{13}C NMR dipolar dephasing experiments in order to assess the degree of substitution for the aromatic rings of intact suberin.

A conventional CPMAS spectrum of the labeled suberin is displayed in Figure 5 (top), revealing very substantial isotopic enrichment of the aromatic carbons

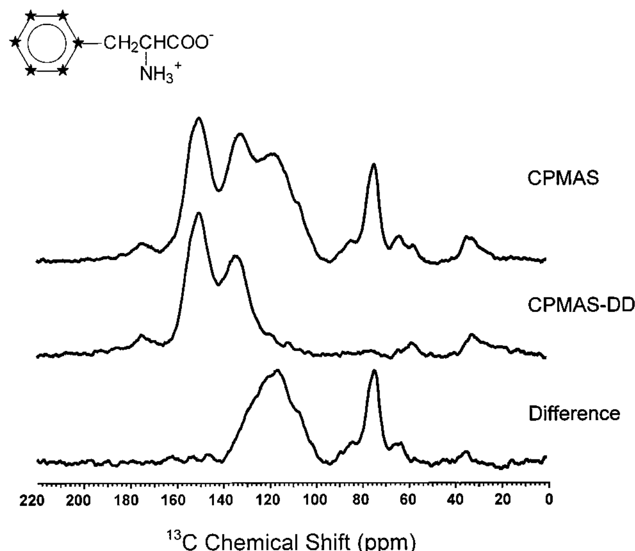


Figure 5. CPMAS ^{13}C NMR spectra of suberin from potato periderm soaked in a solution of [*ring*-$^{13}C_6$]Phe and grown for 7 days: (top) conventional spectrum displaying all aromatic carbons (105−160 ppm); (middle) dipolar-dephased (CPMAS-DD) spectrum showing nonprotonated (quaternary) carbons; (bottom) difference spectrum yielding protonated (methine) carbons. The peak at 150 ppm, which arises solely from nonprotonated carbons and is not overlapped with other resonances, is assumed to have the same intensity in the top and middle spectra. Peak areas for the protonated aromatic resonances are underestimated by ∼10% since they cross-polarize more efficiently and then lose some intensity due to proton spin relaxation (Stark and Garbow, 1992).

that resonate between 105 and 160 ppm (compare the control spectra in the middle of Figures 1 and 3). Nevertheless, definitive identification of the ring structures from their ^{13}C chemical shifts is precluded by the breadth of the spectral features. The corresponding dipolar-dephased spectrum (Figure 5, middle) retains signals from nonprotonated carbons at the substituted positions of the aromatic ring (Opella and Frey 1979) (and from the most flexible aliphatics; Stark et al., 1989). Since the ^{13}C chemical shifts of protonated aromatic carbons usually occur below 140 ppm, it is safe to assume that the peak near 155 ppm arises exclusively from nonprotonated carbons. Thus, we can construct a difference spectrum (Figure 5, bottom) in which these latter resonances are adjusted to zero intensity. The remaining signals at 110−135 ppm are then attributed to protonated aromatic carbons, whereas the peak near 72 ppm reflects mainly contributions from CH carbons of the polysaccharides associated with suberized tissue.

Figure 6 summarizes the possible ring structures, which have predicted signal intensity ratios of protonated (CH) to nonprotonated carbons that range from 0.5 (sinapyl) to 2.0 (*p*-coumaroyl). Since the observed CH/C ratio is 0.7, the suberin aromatic ring structures must consist primarily of sinapyl and guaiacyl moieties. In fact, a calculation of the possible distributions of the three ring structures shows that the *p*-coumaroyl structures cannot exceed 8%, leaving guaiacyl and sinapyl contributions of at least 44% and 48%, respectively. The solid-state ^{13}C NMR experiments thus allow choices to be made among the likely aromatic ring structures in suberin. The present finding of highly substituted guaiacyl and sinapyl structures, when considered in conjunction with prior thioacidolysis degradation analyses (Lapierre et al., 1996), provides clear experimental

	p-Coumaroyl	*Guaiacyl*	*Sinapyl*	*Suberin* (*Guaiacyl and Sinapyl*)
		OCH_3	OCH_3	
CH/C	4/2	3/3	2/4	2.4/3.5
Ratio	2.0	1.0	0.5	0.7 (observed)

Figure 6. Suberin phenolic ring structures and their corresponding ratios of protonated and nonprotonated carbons. The value derived from dipolar-dephasing experiments (Figure 5) implicates a preponderance of guaiacyl and sinapyl moieties.

verification for the frequently stated hypothesis that the suberin polymer possesses a densely cross-linked aromatic architecture.

Several caveats should be noted regarding this quantitation method (Alemany et al., 1983). First, differing cross-polarization efficiencies for the CH and C aromatic carbon types could produce unequal ^{13}C signal sensitivities; prior NMR measurements on suberin indicate that peak areas for the CH resonances are underestimated by ∼10% when the data are acquired using a 1.5-ms contact time (Stark and Garbow, 1992). Second, unequal mobilities and values of the proton rotating-frame relaxation time could compromise the reliability of quantitative determinations, but a common value of 5.3 ms is found for potato suberin (Stark and Garbow, 1992). Finally, the dipolar-dephasing delay time must be chosen to be long enough to eliminate the CH signals (the 72 ppm polysaccharide peak in our spectrum) but not so long that signals from the nonprotonated carbons are also attenuated.

Spin Exchange Involving Suberin Phenolics. Although spin communication on a 5-nm length scale has been demonstrated between separate suberin and cell wall domains (Stark and Garbow, 1992; Yan and Stark, 1998), hypotheses regarding their covalent connections (Kolattukudy, 1980) should withstand direct evaluation of proximal molecular interactions among aliphatic, aromatic, or glycoside moieties of the biopolymer assembly. In a similar fashion, suggestions that isotopically labeled precursors produce non-ester cross-links to aromatic suberin monomers (Bernards et al., 1995) require more direct verification. The detection of heteronuclear through-bond (scalar) connectivities in solid-state NMR is currently limited to one-bond and two-bond interactions (Lesage et al., 1998), but it is possible to examine the predominantly through-space (dipolar) interactions between ^{13}C nuclei at particular chemical sites separated by less than 1 nm.

A series of experiments was conducted on suberin in which the aromatic rings were ∼10% ^{13}C labeled, as judged from a comparison of integrated intensities across the CPMAS ^{13}C NMR spectra of enriched and natural-abundance potato periderm samples. Figure 7 summarizes the results from several two-dimensional CPMAS $^{13}C-^{13}C$ spin-exchange (NOESY) experiments (Szeverenyi et al., 1982; Frey and Opella, 1984; Bardet et al., 1997), where proton-driven spin diffusion between magnetically distinct nuclei is detected by the appearance of off-diagonal (cross) peaks. If the mixing time is omitted, no cross-peaks appear in the spectrum, as expected. As the mixing time is increased to 0.5 and 1.0

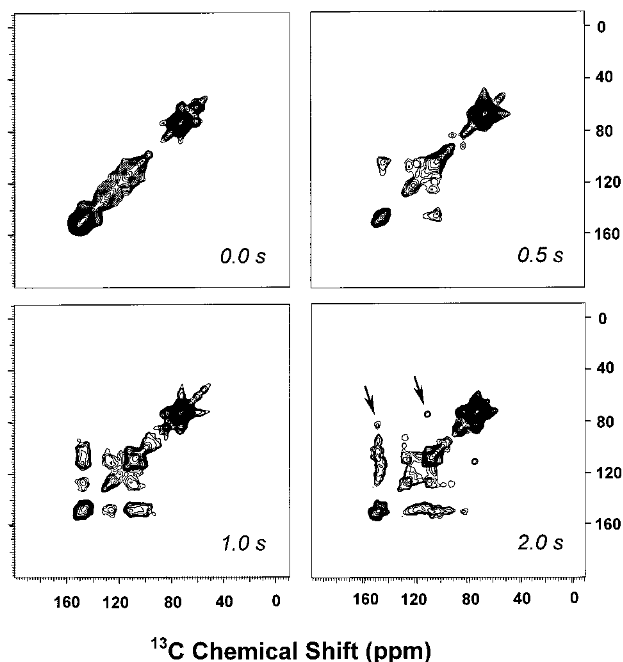


Figure 7. Contour plots of 2D $^{13}C-^{13}C$ spin-exchange data for [*ring*-$^{13}C_6$]phenylalanine-labeled suberin, obtained with mixing times of 0−2 s. Peaks at positions on the diagonal (*n*,*n*) show the conventional 1D ^{13}C CPMAS spectrum, whereas off-diagonal (cross) peaks (*n*,*m* and *m*,*n*) signify pairwise through-space proximities for ^{13}C nuclei with chemical shifts *m* and *n*. The arrows highlight cross-peaks attributable to covalent bonds between the suberin aromatic rings and cell wall glycosides, as described in the text.

s, strong cross-peaks grow in size and number between pairs of the resonances at 110, 124, and 148 ppm, most likely reflecting efficient spin exchange between directly bound, isotopically enriched ^{13}C sites of the aromatic ring. With a mixing time of 2.0 s, two modest but definitive connectivities develop for pairs of carbon resonances at 74, 110 ppm and 82, 148 ppm. The downfield signals (110 and 148 ppm) are assigned to the isotopically enriched guaiacyl (or sinapyl) moieties, on the basis of both an extensive database of ^{13}C chemical shifts and the dipolar-dephasing results presented in Figure 5. The upfield partners (74 and 82 ppm) are oxymethine carbons, which could be attributed to an oxidative coupling product of ferulic acid (Bernards et al., 1995) or to cellulosic cell wall structures. The possibility that these connectivities originate solely from cell wall polysaccharides may be ruled out, since they fail to appear if the $^{13}C-^{13}C$ NOESY experiment is conducted on a sample without aromatic ring labels (data not shown).

Quantitative interpretations of $^{13}C-^{13}C$ spin-exchange experiments are complicated by the presence of natural-abundance nuclei and the use of MAS, but calculations for organic solids (Szeverenyi et al., 1982) and measurements on aspen wood samples (Bardet et al. 1997) make it possible to estimate a length scale of less than 1 nm corresponding to the detection of $^{13}C-^{13}C$ NOESY cross-peaks after a 2.0-s mixing period. On the basis of cross-peaks observed between enriched aromatic ^{13}C's and nearby natural-abundance carbons in crystalline phenylalanine (data not shown), it is further estimated that the phenolic and oxymethine carbons in amorphous potato wound periderm are separated by distances of less than 0.5 nm.

Which of the plausible chemical structures are consistent with the spin diffusion results and this distance estimate? Since H_2O_2/peroxidase-mediated polymerization is involved in suberin formation, oxidative coupling products of ferulic acid such as **3** and **4** have been proposed along with hydroxycinnamic acids (**2**) to account for enhanced ^{13}C NMR signals appearing after incorporation of [2-^{13}C]Phe and [3-^{13}C]Phe (Bernards et al., 1995) (see Scheme 1). Cross-peaks to resonances at 121 and 142 ppm (structure **2**) would be difficult to identify in the presence of strong signals from ring-labeled suberin moieties, but the absence of the expected cross-peaks at 75, 135 ppm and 85, 135 ppm argues against the presence of structure **3** in our suberin preparations. Similarly, the absence of the expected cross-peaks at 86, 135 ppm and 55, 135 ppm argues against the presence of structure **4**. Alternatively, if the cross-peaks involve glycosidic carbons, then they provide evidence of close proximity between the phenolic moieties of suberin (110 and 148 ppm) and the cell wall polysaccharides (72 and 83 ppm), as shown in **5**. This latter hypothesis supports the earlier suggestion that suberin is anchored to the cell wall at discrete sites, as judged from spin-relaxation data showing that suberin methylenes and aromatics each have rigid and mobile carbon populations (Stark and Garbow, 1992).

In summary, new molecular-level information regarding the biosynthesis and architecture of suberizing potato tissue has been obtained through the combined use of selected ^{13}C-enriched precursors and solid-state CPMAS ^{13}C NMR. In addition to isotopic labeling that enhances the structural and mechanistic usefulness of direct spectroscopic observations in this intractable plant biopolymer system, our investigative approach uses spectral editing to identify proposed phenolic moieties and two-dimensional spin exchange to evaluate the possibility of polyester−polysaccharide bonds. Our findings reveal both polyester-forming *p*-hydroxycinnamic acids and lignin-like monolignols that constitute a dense, covalently cross-linked network capable of repelling water (Yan and Stark, 1998) and protecting the cell wall polysaccharides from pathogenic attack.

The biosynthesis of long-chain fatty acids within suberin is likely to follow the conventional pathway of two-carbon additions using malonyl-CoA, but incorporation of site-specifically ^{13}C-labeled acetates into aliphatic structures of the developing suberin polymer occurs preferentially at the carboxyl end of the acyl chain. This "uneven" pattern of isotopic enrichment suggests that exogenous precursors are involved only in the later elongation steps of fatty acid synthesis that take place on the endoplasmic reticulum, and it argues for cautious interpretation of ^{13}C signal intensities in terms of which molecular constituents are present or how much chain labeling has occurred.

The incorporation of phenylalanine into the aromatic residues of suberizing potato tissue produces both monolignols and *p*-hydroxycinnamic acids, identified provisionally through the use of carboxyl-labeled precursors. Our NMR spectra reveal roughly equal amounts of these aromatic constituents, in contrast to both GC−MS studies of soluble suberin depolymerization products (Cottle and Kolattukudy, 1982) and CPMAS ^{13}C NMR reports for intact potato wound periderm (Bernards et al., 1995). The question of which structure is predominant may hinge on how the precursors are administered and/or how the suberin samples are isolated, although

no definitive explanation is currently available. Our solid-state NMR experiments show that both acid and monolignol structures develop concurrently, within a 5-day time frame matched by the development of diffusion resistance in potato wound periderm. CPMAS ^{13}C NMR with dipolar dephasing indicates that the aromatic ring structures consist primarily of substituted phenolics such as guaiacyl and sinapyl moieties. This finding provides the most direct experimental verification to date of the highly cross-linked architecture of suberin.

Finally, the most plausible interpretation of $^{13}C-^{13}C$ spin diffusion experiments in suberized potato periderm places the isotopically labeled aromatic carbons of suberin and glycosidic carbons of the cell wall polysaccharides in close proximity to one another. Distance estimates of 0.5 nm have been made by reference to analogous experiments on wood and phenylalanine samples. These findings support the longstanding hypothesis of covalent bonding between suberin and cell wall biopolymers (Kolattukudy, 1980), and the NOESY cross-peaks observed in the current study implicate chemical bonds between the guaiacyl or synapyl rings of suberin and the cell wall glycosides. Additional efforts are ongoing in our laboratory to confirm and extend these structural conclusions using high-resolution MAS NMR of solvent-swelled suberin preparations.

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