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Altered lignin composition in phenylalanine ammonia-lyase-inhibited radish seedlings: implications for seed-derived sinapoyl esters as lignin precursors

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Abstract

We earlier reported that when phenylalanine ammonia-lyase (PAL) activity in radish seedlings was inhibited by the competitive inhibitor 2-aminoindan-2-phosphonic acid (AIP), soluble sinapoyl esters carried over from the seed were converted to wall-bound esters in young cotyledons. We now report that these soluble sinapoyl esters may also be converted into lignin in the cotyledons. When radish seedlings were grown in the presence of 100 μM AIP, lignin formation (determined as lignothioglycolic acid) was inhibited ca. 74% in the cotyledons and ca. 80% in hypocotyls plus roots. The syringyl to guaiacyl (S/G) ratio in the lignin of AIP-grown plants, as determined by alkaline cupric oxidation and from Fourier-transform infrared (FT-IR) spectra, was higher in cotyledons, but lower in hypocotyls plus roots, as compared to plants grown on distilled water. These results support the view that soluble sinapoyl esters preformed in seeds may contribute to the syringyl moiety of lignin in cotyledons during early seedling development and that there is no appreciable transport of soluble sinapoyl esters from cotyledons to the hypocotyls and roots.

Keywords: *Raphanus sativus*; Cruciferae; Radish; Sinapoyl esters; 2-Aminoindan-2-phosphonic acid (AIP); Phenylalanine ammonia-lyase (PAL); Lignin; Lignothioglycolic acid (LTGA); Fourier-transform infrared spectroscopy (FT-IR)

1. Introduction

During the first week of radish seedling growth, levels of seed-derived sinapine (sinapoylcholine) and 6-sinapoylglucoraphenine decrease ca. 50% and about one percent of these sinapoyl esters can be accounted for by esterification to cell walls in the developing seedling (Chen, Gitz & McClure, 1998). We are unaware of any reports of the metabolic degradation of soluble sinapoyl esters, but several authors have proposed that they might serve as precursors for lignin (Strack, 1977; Amrhein, Frank, Lemm & Luhmann, 1983; Chapple, Shirley, Zook & Hammerschmidt, 1994). Further, recent reports suggest that transgenic *Arabidopsis*, another member of the Cruciferae, with reduced activity for either L-phenylalanine ammonia-lyase (PAL) or 4-coumarateCoA ligase (4CL) showed decreased levels of guaiacyl residues in lignin but little change in levels of syringyl residues. This led us to examine radish seedlings to determine if growth with AIP increased the ratio of sinapoyl to guaiacyl moieties in the lignin, as would be expected if seed-derived sinapoyl esters were converted into lignin in plants with diminished PAL activity.

Growth with 100 μM 2-aminoindan-2-phosphonic acid (AIP), a competitive inhibitor of PAL (Zon & Amrhein, 1992; Nakashima, Takabe, Fujita & Saiki, 1997), produces radish seedlings whose cotyledons have high levels of wall-bound sinapoyl esters but only traces of wall-bound ferulic acid. Since this also almost completely inhibits formation of wall-bound sinapic

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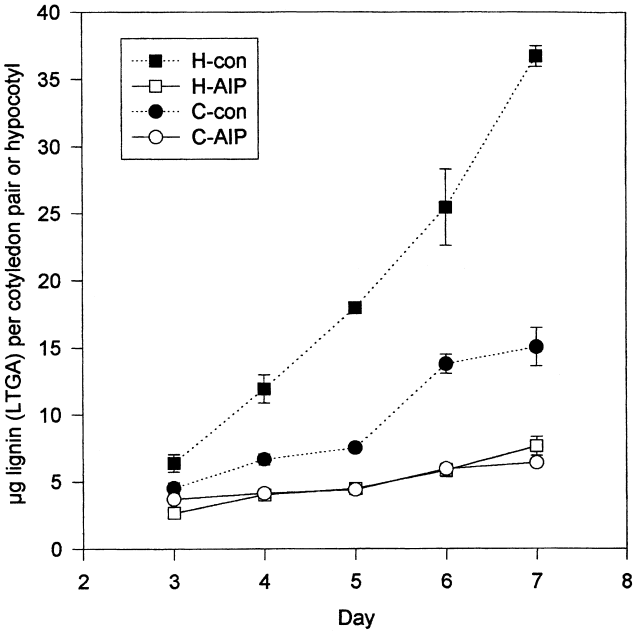

Fig. 1. Changes in lignin as lignothioglycolic acid (LTGA) from radish seedlings grown on filter paper with distilled water (con.) or 100 μM 2-aminoindan-2-phosphonic acid (AIP) at 23°C. After two days in the dark seedlings were transferred to 300 μM PAR from cool-white fluorescent lights. Extractive-free cell walls were prepared, and LTGA extracted and assayed, as described in Section 4. C = cotyledon pair, H = hypocotyl plus roots, con. = control (distilled water). Values are means ± S.E.; $n = 3$.

and ferulic acids in hypocotyls plus roots, we grew radish seedlings on either 100 μM AIP or on distilled water, and these were separated at harvest into cotyledon and hypocotyls plus roots. We hypothesized that plants whose de novo phenolic biosynthesis was inhibited by AIP would have diminished ability to produce precursors for guaiacyl units of lignin, but would still be able to utilize soluble seed-derived sinapoyl esters as precursors for syringyl units of lignin, and thus increase the ratio of syringyl to guaiacyl units (S/G ratio) in the lignin polymer. To detect such possible cycling of soluble sinapoyl esters into lignin, the S/G ratio of lignin from AIP-grown and control plants was determined by alkaline cupric oxidation and by FT-IR spectroscopy of lignothioglycolic acid (LTGA) preparations, respectively.

2. Results

Fig. 1 shows developmental changes in lignin content in cotyledons and hypocotyls plus roots, of radish seedlings grown on distilled water (control) or on 100 μM AIP. Between day 3 and day 7, AIP inhibited lignin formation ca. 74% in cotyledons and 80% in hypocotyls plus roots.

Table 1 shows lignin-derived benzaldehydes from cotyledons and hypocotyls plus roots of plants grown on distilled water or on 100 μM AIP. In control cotyledons, guaiacyl (oxidized to vanillin) was the dominant structural unit but the syringyl (oxidized to syringaldehyde) to guaiacyl ratio (S/V = S/G) increased from 0.06 to 0.17 between day 3 and day 7. Growth with AIP inhibited accumulation of guaiacyl units sightly more than syringyl units and in these plants the S/V ratio changed from 0.01 to 0.21 between day 3 and day 7, suggesting that seed-derived sinapoyl esters contributed to syringyl residues in these plants. In hypocotyls plus roots grown on distilled water, the S/V ratio increased from 0.22 to 0.92 between day 3 and day 7, but in AIP-grown plants this ratio varied only from 0.08 to 0.11 during this time, suggesting appreciable de novo synthesis of syringyl precursors in these organs of control plants but little, if any, in AIP-grown plants.

To confirm that the S/G ratio increased more in cotyledons grown on AIP than on distilled water, LTGAs were extracted from both groups and their FT-IR spectra determined. In these spectra (Fig. 2)

Table 1
Effect of AIP on lignin monomer composition in radish seedlings grown as in Fig. 1. Extractive-free cell walls were subjected to CuO oxidation and the lignin-derived benzaldehydes vanillin (V) and syringaldehyde (S) determined by HPLC. Control = grown with distilled water, AIP = grown with 100 μM AIP. S/V = syringaldehyde/vanillin ratio. Since vanillin (V) is formed by oxidation of guaiacyl (G) units in lignin, S/V correlates with S/G of other workers. Units are nmole per cotyledon pair or hypocotyl plus roots. Values are means ± S.E.; $n = 3$

Day	Control			AIP		
	S	V	S/V	S	V	S/V
			Cotyledons			
3	1.01 ± 0.01	17.78 ± 0.06	0.06	0.16 ± 0.08	17.11 ± 1.53	0.01
5	5.35 ± 0.22	38.33 ± 2.26	0.14	2.60 ± 0.24	19.93 ± 0.51	0.13
7	9.39 ± 0.58	56.38 ± 0.14	0.17	5.67 ± 0.17	26.79 ± 3.57	0.21
			Hypocotyl plus roots			
3	4.51 ± 0.60	20.07 ± 1.07	0.22	0.54 ± 0.26	7.07 ± 0.79	0.08
5	18.38 ± 1.28	52.59 ± 4.93	0.35	2.76 ± 0.14	26.79 ± 1.34	0.10
7	87.43 ± 18.69	94.10 ± 7.43	0.93	3.54 ± 0.71	32.62 ± 5.45	0.11

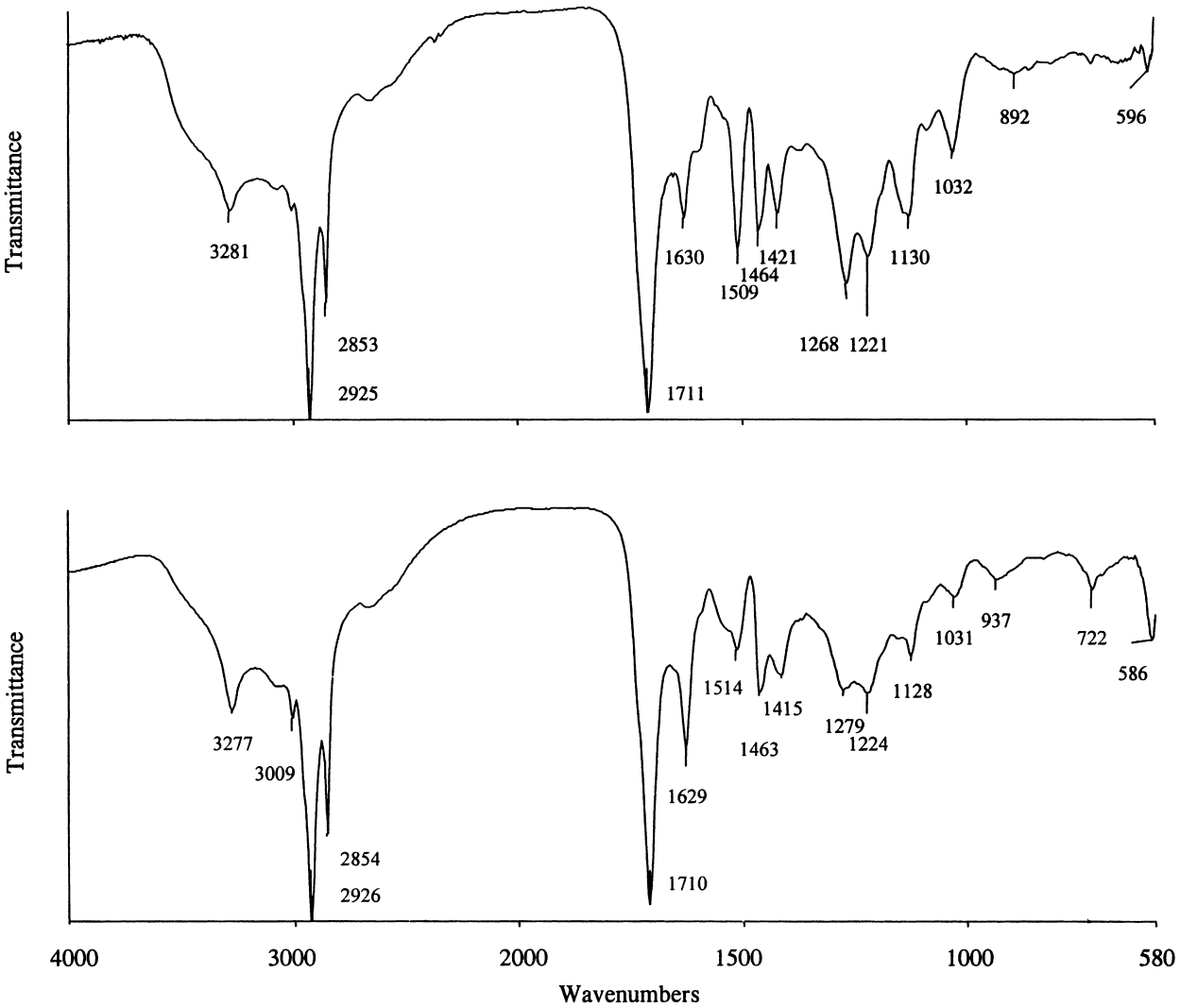

Fig. 2. FT-IR spectra of LTGA from 7-day-old radish cotyledons. Plants were grown on either distilled water (upper spectrum) or on 100 μM AIP (lower spectrum). In these spectra the 1268 and 1279 cm^{-1} bands are associated with guaiacyl units of lignin and the 1221 and 1224 cm^{-1} bands with syringyl units of lignin (see Section 3). Note the enrichment of syringyl units in plants grown with AIP.

bands at 1509, 1421, 1514, and 1415 cm^{-1} are aromatic skeletal vibrations, bands at 1464 and 1463 cm^{-1} are from C–H deformations related to methyl groups, bands at 1268 and 1279 cm^{-1} are associated with the guaiacyl moiety, and the bands at 1221 and 1224 cm^{-1} are associated with the syringyl moiety (Hergert, 1971; Stewart et al., 1997; Jung & Himmelsbach, 1989; Faix, 1991). Faix (1991) reviewed lignin FT-IR spectra and concluded that relative S/G ratios can be deduced by comparing relative absorption intensities of peaks at ~1462 cm^{-1} to ~ 1510 cm^{-1}, and ~1220 cm^{-1} to ~1270 cm^{-1}. A high S/G ratio is correlated with a high ratio of peak intensities at ~1462 cm^{-1} compared to ~1510 cm^{-1}, and ~1220 cm^{-1} compared to ~1270 cm^{-1} (Faix, 1991). It is evident (Fig. 2) that lignin from 7-day-old cotyledons grown on AIP has a higher S/G ratio than does lignin from plants grown on distilled water.

The S/G ratios determined by CuO oxidation and by FT-IR both support the view that seed-derived soluble sinapoyl esters can be incorporated into lignin of cotyledons, but probably not in hypocotyls plus roots, during early stages of growth.

3. Discussion

Little information is available about lignin of young plants of the Cruciferae. Syringaldehyde and vanillin were not detected after nitrobenzene oxidation of cell wall preparations from 4-day-old *Arabidopsis* seedlings, although histochemical examination indicated that lig-

nification in these plants started about 36 h after germination (Dharmawardhana, Ellis & Carlson, 1992).

In the cotyledons of 3-day-old plants levels of lignin as LTGA (Fig. 1) and as lignin oxidation products (Table 1) are very similar in plants grown on either distilled water or on 100 μM AIP, but in hypocotyls plus roots, plants grown on distilled water have more than twice as much lignin as plants grown on 100 μM AIP (Fig. 1, Table 1). This may indicate that lignification starts much earlier in hypocotyls plus roots than in the cotyledons. Alternately, pools of lignin precursor carried over from the seed into the expanding cotyledons may provide precursors for the earliest stages of lignification.

Between day 3 and day 7, growth with 100 μM AIP inhibits lignin formation as LTGA ca. 74% in the cotyledons and ca. 80% in hypocotyls plus roots (Fig. 1). This is consistent with the view that some of the cotyledon lignin was produced from seed-derived sinapoyl esters. This was supported by the CuO oxidation results. In CuO oxidation sinapoyl (syringyl) units are converted to syringaldehyde (S) and coniferyl (guaiacyl, G) units are converted to vanillin (V), thus the ratio of S/V is equivalent to S/G. Note that when these seedlings were grown on distilled water their S/G ratio increased from 0.06 to 0.17 (2.8 fold) in cotyledons and from 0.23 to 0.92 (4.2 fold) in hypocotyls plus roots. In contrast, when grown on AIP the S/G ratio increased from 0.01 to 0.21 (21 fold) in cotyledons, but only from 0.08 to 0.11 (1.4 fold) in the hypocotyls plus roots.

When grown on 100 μM AIP, soluble sinapoyl esters decrease ca. 15 μg (ca. 67 nmol, as sinapic acid) per cotyledon pair between the third and seventh day of radish seedlings growth and ca. 0.45 nmole of these soluble esters are converted to wall-bound esters (Chen et al., 1998).

It is not likely that wall-bound esters of sinapic and ferulic acids that may have been left in our extractive-free cell walls contributed much to the pool of syringaldehyde and vanillin recovered after alkaline cupric oxidation. Assuming a recovery of about 20% of the syringyl and guaiacyl units in lignin as syringaldehyde and vanillin after CuO oxidation (Lewis & Yamamoto, 1990), the contribution from wall-bound sinapic and ferulic acids would be less than 0.09 and 0.18 nmol, respectively, well within the error term of recovered benzaldehydes (Table 1). Similarly, Musel et al. (1997) reports that LTGA preparations are free from wall-bound phenolic esters, as might be expected for samples which have undergone the rigorous hydrolysis involved in LTGA preparation, and we find no bands characteristic of ester linkages in our FT-IR spectra of LTGA (Fig. 2).

CuO oxidation of lignin also produces good yields of benzoic acids, e.g. vanillic, in addition to aldehydes, e.g. vanillin. However, since most who have used this technique to characterize lignin report only the recovery of the benzaldehydes (Dharmawardhana et al., 1992; Lewis & Yamamoto, 1990; Sewalt et al.; Lee, Meyer, Chapple & Douglas, 1997), and with the goal of relating our work to that of others, no attempt was made to quantitate or characterize the benzoic acids.

Much evidence has supported the view that monolignol glucosides (e.g. coniferin) serve as storage forms of lignin precursors (Lewis & Yamamoto, 1990; Barcelo, 1997; Davin & Lewis, 1991). Radish seeds contain large amounts of 6,3-disinapoylsucrose and sinapoylcholine which are rapidly converted to 1-sinapoylglucose (Linscheid, Wendisch & Strack, 1980) which we suggest is a likely candidate for subsequent metabolic conversion to a monolignol glucoside.

It has recently been reported that inhibition of PAL or 4CL activity in tobacco (Sewalt et al.), and 4CL in *Arabidopsis* (Lee et al., 1997), by either sense or antisense suppression, results in a reduction in guaiacyl units in lignin with little or no effect on its syringyl content. The authors suggested that there may be an uncharacteristic route to sinapoyl alcohol which is independent of PAL and 4CL, although no suggestions have been provided about what this pathway might be. We cannot rule out the existence of some unknown metabolic pathway to explain our results with AIP. However, our results do indicate that if such a pathway occurs in radish it seems to operate in cotyledons but not in the hypocotyls plus roots, since growth with AIP increases the S/G ratio 21 fold in cotyledons, but only 1.4 fold in hypocotyls plus roots, between days 3 and 7 (Table 1). To further investigate this phenomenon, we are extending our work to a study of AIP effects on lignin levels and composition in mature leaves of 30 day old radish plants, which should have no carry over of sinapoyl esters derived from the seed.

4. Experimental

4.1. Plant materials and growth conditions

Radish seeds, *Raphanus sativus* L. cv Cherry bell, were purchased from W.A. Burpee Co. (Warminster, PA) and grown as previously described (Chen et al., 1998). Briefly, seeds were placed on filter paper in 10 cm diameter petri dishes, moistened with distilled water or 100 μM AIP and grown at 23°C. They were kept in the dark for the first two days to facilitate germination and hypocotyl elongation, and then transferred to continuous 200 μM PAR from cool white fluorescent lamps. At harvest cotyledons were separated from the hypocotyl plus roots by cutting at the node.

4.2. Preparation of extractive-free tissues

Radish seedlings, especially the cotyledons, contain considerable protein which, unless it is removed before LTGA is extracted, coprecipitates with lignin and causes gross overestimation of lignin based on the absorbance of LTGA at 280 nm (Chen, Sommer & McClure, in press). To resolve this problem we have modified the conventional procedure for preparing extractive-free tissue from herbaceous samples, which is usually initiated by extraction with MeOH or EtOH (Lee et al., 1997; Dean, 1997), as follows: Cotyledons or hypocotyls plus roots were homogenized in 50 mM K-phosphate (pH 7.0) buffer with a mortar and pestle and washed by stirring and centrifugation with, successively, pH 7.0 K-phosphate buffer (3×), 1% (v/v) Triton X-100 in pH 7.0 buffer (3×), 1M NaCl in pH 7.0 buffer (2×), distilled water (2×) and acetone (2×). The resultant cell wall fraction was dried in a vacuum desiccator. This procedure removes most of the protein from the samples and produces extractive-free cell walls which, based on recovery of lignin-derived benzaldehydes, does not cause appreciable loss of lignin from the samples (Chen et al., in press).

4.3. Quantitation of lignothioglycolic acid (LTGA)

Following procedures of Dean (1997), extractive-free tissue (about 15 mg) was placed in a screw-cap centrifuge tube with 1 ml of 2 M HCl and 0.2 ml of thioglycolic acid and heated for 4 h at 95°C. The sample was cooled to room temperature, centrifuged, and the supernatant discarded. After washing with distilled water (3×), lignothioglycolate was extracted by vigorous shaking at 30°C for 18 h in 1 ml of 0.5 M NaOH. After centrifugation, the supernatant was reserved and combined with supernatant from washing the pellet with 0.5 ml of 0.5 M NaOH. The combined alkali extracts were acidified with 0.3 ml of concentrated HCl and the LTGA precipitate formed after 4 h at 4°C recovered by centrifugation and washed twice with distilled water. The LTGA pellet was dissolved in 0.5 M NaOH and diluted with 0.5 M NaOH to yield an appropriate absorbance at 280 nm. An absorption coefficient of 6×10^{-3} ml/g cm was used for LTGA quantitation (Dean, 1997).

4.4. Alkaline cupric oxidation

Extractive free tissue (about 15 mg) was sealed in a stainless steel Parr bomb with 1 ml of 2 M NaOH plus 200 mg of CuO and heated to 170°C for 2 h, shaking the sample occasionally during the reaction. After oxidation the sample cooled, acidified with 2 M HCl to about pH 2 and extracted with Et_2O (3×). The Et_2O fraction was taken to dryness under N_2, and the residue dissolved in 1 ml of MeOH for HPLC.

4.5. HPLC detection of alkaline cupric oxidation degradation products

Lignin-derived benzaldehydes were assayed by HPLC as previously described for wall-bound phenolics (Chen et al., 1998) using the same C18 RP column and isocratic solvent of 1% (v/v) aqueous H_3PO_4–MeOH–tetrahydrofuran (67:21:12) at a flow rate of 0.7 ml/min. Absorbance was monitored at 290 nm and syringaldehyde and vanillin were identified by co-injection with authentic compounds. Further confirmation of the identities of the benzaldehydes was established using the TLC system described by Blazey and McClure (1968). Quantitation was based on peak area compared with external standards of authentic compounds.

4.6. Preparation of LTGA for determining FT-IR spectra

Extractive-free tissue (prepared as above) from 200 pairs of cotyledon of 7-day-old seedlings grown on distilled water or on 100 μM AIP, was incubated with 25 ml of 20 mM, pH 5.7, K-phosphate containing 2% (w/v) Cellulysin (Calbiochem, La Jolla, CA) and 0.2% (w/v) Pectolyase Y-23 (Seisin Pharmaceuticals, Tokyo, Japan) in a 1.5 ml screw-cap vial and shaken for 48 h at 30°C. The digested tissue was centrifuged at 16,000 g for 10 min, the supernatant discarded, and the pellet washed by stirring and centrifugation with 20 mM K-phosphate (pH 5.7) buffer (3×) and finally with distilled water (3×). This additional treatment removed much of the non-lignified cell wall material from the samples and yielded LTGA whose FT-IR spectra were comparable to that of LTGA prepared from Kraft hardwood lignin (Chen et al., in press). LTGA was extracted from these pellets as described above, and weights and volumes were adjusted accordingly.

4.7. Fourier-transform infrared (FT-IR) spectroscopy

Infrared spectra were collected with a Perkin–Elmer Spectrum 2000 FT-IR equipped with a Perkin Elmer FT-IR infrared microscope. Detection of the infrared radiation was afforded by 250 × 250 μm, liquid nitrogen cooled HgCdTe detector. Samples were prepared for analysis by flattening a small amount on a potassium chloride salt plate with the aid of a fine pointed probe. A cross-sectional area of approximately 50 × 50 μm was analyzed for each sample. Each spectrum represents the average of 64 individual scans collected at 4 cm^{-1} resolution.

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