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Molecular Structure of Humin and Melanoidin via Solid State NMR

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Abstract

Sugar-derived humins and melanoidins figure significantly in food chemistry, agricultural chemistry, biochemistry and prebiotic chemistry. Despite wide interest and significant experimental attention, the amorphous and insoluble nature of the polymers has made them resistant to conventional structural characterization. Here we make use of solid-state NMR methods, including selective ¹³C substitution, ¹H-dephasing, and double quantum filtration. The spectra, and their interpretation, are simplified by relying exclusively on hydronium for catalysis. The results for polymers derived from ribose, deoxyribose and fructose indicate diverse pathways to furans, suggest a simple route to pyrroles in the presence of amines, and reveal a heterogeneous network-type polymer in which sugar molecules cross-link the heterocycles.

Keywords

sugar polymerization; sugar dehydration; microspherules; furan; pyrrole; crosslinks; Maillard; Amadori

INTRODUCTION

The “browning reaction” of sugars in the presence of amino acids, or organic salts of amines, has long been of interest in food chemistry, biochemistry and agricultural chemistry.^{1,2} However, despite its ubiquity, the structures of the resulting “melanoidin” polymers remain unknown. This is because the amorphous and insoluble nature of the polymers makes them unsuited to direct study by either diffraction or solution NMR. Instead MALDI-TOF-MS, GC/MS and solution NMR studies have targeted model reactions, on the basis of which Tressl *et al.*^{3–5} have proposed linear and branched polymers in which bridging carbons link furan and pyrrole units as shown in Chart 1.

Interest in melanoidin has recently extended to prebiotic chemistry, as the polymer can form gelatinous globules that have the potential to compartmentalize and facilitate proto-metabolism by reducing the diffusion of metabolic intermediates.^{6,7} IR analyses show that these “microspherules” also contain furans and pyrroles,⁶ when formed from erythrose in the presence of ammonium acetate. Recently, we have observed the formation of similar

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microspherules without amines, i.e., under conditions associated with the formation of humins.⁸ This observation suggests that humins and melanoidins are structurally similar. It also offers an opportunity to use solid state NMR (ssNMR) to characterize the structure of the amorphous polymer because the heterocycle portion of the spectrum is greatly simplified by the absence of pyrrole signals. Here we present variously edited ¹³C ssNMR spectra of samples formed from selectively labeled sugars and show that the furans produced in browning are embedded in complex hetero-polymers, rather than homo-polymers of the sort proposed by Tressl *et al.*^{4,5}

EXPERIMENTAL SECTION

Materials

The sugars D-ribose, D-fructose, D-glucose, D-galactose, and D-mannose were obtained from Sigma-Aldrich; 2-deoxy-D-ribose from Acros Organics; and ¹³C-labeled 2-deoxy-D-ribose, D-ribose, and D-fructose from Omicron Biochemicals.

Reaction conditions

Polymers for ssNMR analysis were prepared from initially dry reactants to obtain high yields. Equimolar mixtures of sugar and oxalic acid were ground together in a mortar and sealed in a glass vial. Sugars included D-ribose, 2-deoxy-D-ribose, D-fructose, D-glucose, D-galactose, and D-mannose. Control samples were also prepared for each sugar with no oxalic acid. After incubation at 65°C for 2–9 weeks, the insoluble product was washed four times with de-ionized water, dried, and sealed in another vial under argon.

NMR Spectroscopy

Samples (typically 50 mg) were loaded into 4 mm zirconia rotors under a dry nitrogen atmosphere. All spectra were recorded at 360 MHz (¹H frequency) using a home-built spectrometer and software (D. Ruben, MIT) with a Varian triple-resonance probe equipped with a solenoid-coil 4 mm magic-angle spinning (MAS) system at room temperature. The MAS frequency was controlled at 7, 8 or 10 kHz $\pm$ 3 Hz for a given experiment to avoid spinning sideband overlap with signals. The spectral width was set to about 100 kHz for all measurements and the FID was recorded for ~5.4 ms. The chemical shifts were referenced to TMS using the ¹³C signals of adamantane (38.48 and 29.46 ppm) as standards.

Data were accumulated in blocks of 560 transients with 3 seconds recycle delays, and added together before Fourier transformation. The blocks allow us to check the stability of the signal over acquisition times extending from 1 to 5 days, and to stop the acquisition when the S/N ratio is good enough. Typically 10 and 50 blocks were acquired for ¹³C CP experiments^{9,10} with two pulse phase modulation (TPPM) decoupling¹¹ on isotope-labeled samples and un-labeled samples experiments, respectively. For ¹³C DQF experiments,¹² 100 blocks were typical.

For ¹H-¹³C dipolar-dephasing experiments, a spin-echo period (delay—pi-pulse—delay) was inserted after the CP; one rotor period (125 μ s) was used for the delay during which the ¹H dipolar decoupling field was turned off. In the ¹³C-¹³C DQF experiment, the double-quantum coherence was excited and reconverged with a 0.80 and 1.14 ms-long SPC5 sequence at 10 and 7 kHz MAS respectively.¹² The reconversion block was phase-cycled as 1234 with the receiver phase 1313 to select the DQ component.

The CP time was 1 ms with a ¹H RF-field strength of 50 kHz. During acquisition and SPC5, the ¹H RF-field strength was 83 and 100 kHz, respectively. For pulses in the SPC5

sequence, the ^{13}C -field strength was 35 and 50 kHz at MAS frequencies of 7 and 10 kHz, respectively.

All data were processed using RNMR (D. J. Ruben, MIT). Prior to Fourier transformation, the free induction decay was zero filled to 1024 points and 100 Hz exponential line broadening was applied.

RESULTS AND DISCUSSION

Reactions

Table 1 shows the reactivity of pentoses and hexoses in the presence of oxalic acid at 65°C. The first indication of reaction is moistening of the initially dry material, presumably due to release of water. Further progress is marked by the formation of dark product. In the control samples without oxalic acid, no dark product is observed within 90 days for any of the sugars. In the presence of oxalic acid, pentoses generally form dark product within hours, as does the ketohexose D-fructose, whereas aldohexoses take days to polymerize.

The yields (i.e., % of sugar captured in dried polymer after four washes with deionized water) follow the same general trend: yields from the pentoses and the ketohexose fructose are higher than yields from the aldohexoses. In particular, the reaction of ribose in the presence of oxalic acid produces polymer with an average 50% yield after 48–68 days; similarly, the yield for the reaction of D-fructose with oxalic acid was approximately 50% after 47 days. The reaction of 2-deoxy-D-ribose with oxalic acid already produces polymer with an approximately 45% yield after only 15 days. However, the yield of the reaction with glucose remained low (approximately 1% after 46 days), and there was not enough solid product from this reaction to be analyzed by ssNMR.

The pattern of sugar reactivity matches the previously reported formation of microspherules in solution,⁸ and its correlation with the occurrence of furanose forms in solution (Table 1) suggests that simple dehydration of these 5-membered heterocycles could be responsible for the furan signals seen in FTIR spectra.⁶ Such an intact ring pathway is the favored route to 5-(hydroxymethyl)-2-furaldehyde (5-HMF) from D-fructose.¹⁶

Solid State NMR

Due to the slow reaction rates of, and low yields from, the aldohexoses we focused our spectroscopy on the polymers produced from D-ribose, 2-deoxy-D-ribose, and D-fructose (henceforth simply ribose, deoxyribose, and fructose, respectively). ^{13}C ssNMR spectra of the polymeric products formed from the unlabeled sugars are shown in Fig. 1.

Furans figure prominently, with the relatively sharp peak at ~150 ppm corresponding to the 2,5-carbons and the broader signal at ~110 ppm corresponding to the 3,4 carbons. The peaks at ~210 ppm, ~175 ppm, and 60–80 ppm show that oxygen is also present within the polymer as ketone, acid or ester, and alcohol or ether, respectively. The presence of acid is consistent with the acid nature of humins. The upfield signals (20–40 ppm) indicate that the polymer also contains methylene carbons, as expected in sugar dehydration.

The observed dephasing by ^1H 's (dotted traces) is consistent with these assignments: ketone (~210 ppm) and acid or ester (~175 ppm), carrying no proton, are not dephased, while alcohols or ethers (60–80 ppm) and methylenes (20–40 ppm) are dephased. Of particular interest is the survival of the 2,5-furan signals (~150 ppm), indicating that both are substituted. In pentoses, only one of these carbons can be attached to a pendant terminal carbon of the original sugar molecule, and the other must be involved in polymerization.

This is consistent with the nucleophilicity of the 2,5-furan carbons and the occurrence of carbocations in acidic media.

If the furan rings arise directly from dehydration of furanose rings, as has been established for fructose,¹⁶ we expect deoxyribose-C1, ribose-C1, and fructose-C2 to appear in the 2,5-furan positions of the polymer. Fig. 2 shows spectra of polymers made from the sugars with ¹³C at those positions. The expected 2, 5-furan signal at ~150 ppm is observed for the polymers from [1-¹³C]-deoxyribose and [2-¹³C]-fructose, along with other signals reflecting a complex product. For [2-¹³C]-fructose, the ketone (~210 ppm) peak indicates that some fructose joins the polymer without affecting the carbonyl at C2. At the same time, the C2 acid-ester peak (~175 ppm) suggests that the known decomposition of 5-HMF to levulinic acid and formic acid¹⁸ has led to incorporation of the former into the polymer. For [1-¹³C]-deoxyribose, the acid-ester signal (~175 ppm) suggests that in some cases dehydration of the furanose ring leads to the cyclic ester, γ -lactone, rather than furan. Other signals indicate that, in some of the molecules incorporated into the polymer, C1 has participated in an aldol condensation transforming C1 into an alcohol (60–80 ppm) or, upon dehydration, a ketone (~210 ppm) or a methylene (20–40 ppm) or a 3,4 carbon of furan (~110).

Fig. 2 tells a rather different story for the polymer produced from [1-¹³C]-ribose: the dominant, relatively narrow peak at ~100 ppm in the spectrum of the polymer produced from [1-¹³C]-ribose suggests that ribose-C1 persists as hemiacetal or acetal (hydrated aldehyde being unlikely under the highly dehydrating conditions). For polymers from ribose, the 2,5-furan signal is actually found in samples prepared from [2-¹³C]-ribose and [5-¹³C]-ribose (Fig. 3). This distribution of carbons has been seen by Nimlos *et al.*¹⁹ in solution NMR studies involving xylose and is the arrangement expected when furans from pentoses arise via the acyclic dicarbonyl intermediate proposed by Ahmad *et al.*²⁰ (consistent with the C2 ketone signal in Fig. 3).

The straightforward pathways to furan for the three sugars (summarized in Scheme 1) suggest that furan will be the dominant heterocycle even when polymerization occurs in the presence of amine. However, if amine is present, protonation of the furan ring could lead to ring opening and replacement of oxygen with nitrogen.^{21,22} Thus a dynamic equilibrium between furans and pyrroles could explain the presence of pyrroles in the polymer when amine is used as co-reactant.

It remains to determine what new bonds are involved in the formation of polymer. The ribose product in Scheme 1 suggests an acid-catalyzed attack of the aldehyde from ribose-C1 (black) at the electron-rich 2,5-furan positions from ribose-C5 (purple). This reaction would give Tressl's branching polymer (as in Chart 1 with X=O).^{4,5} Indeed, we know from the ¹H-dephased ¹³C spectrum in Fig. 1 that the 2,5-furan position is heavily substituted (i.e., carries no proton). However, since ribose-C1 appears only as hemiacetal or acetal in the polymer (Fig. 2), it cannot be the partner for ribose-C5 in the polymer. On the other hand, attack of carbocations at C1 on the abundant electron-rich alcohols of nearby sugars (as shown in Scheme 2) would yield hemiacetals and acetals consistent with the spectrum in the middle of Fig. 2. A similar system has been seen recently in the reaction of the disaccharide trehalose with furfural.²³

The remaining question then is how the ribose-C5 becomes substituted in the furan ring. A clue comes from double-quantum filtering (DQF) experiments, which detect only directly bonded pairs of ¹³C-labeled carbons. In particular, the observation that both 2,5-furan and methylene signals survive in a DQF spectrum of polymers prepared from [5-¹³C]-D-ribose (Fig. 4) suggests that the furans are attacked by carbocations formed in acid-catalyzed dehydration of the abundant alcohols of nearby sugar molecules (as shown in Scheme 3).

Thus, to explain the dominant signals in our spectra of polymers from ribose, we suggest a polymer in which furaldehydes cross-link sugars as shown in Scheme 4. Overall, polymerization could be complex, as each furan unit can react with at least 2 other sugars, and each sugar molecule can react with multiple furan units.

Analogous structures are expected in the polymers from deoxyribose and fructose. In the case of deoxyribose (top of Scheme 1), the furan ring bears an alcohol group rather than an aldehyde and is therefore expected to form an ether linkage with a nearby sugar, rather than the (hemi)acetal formed in the ribose polymer. In the case of fructose (bottom of Scheme 1), the furan ring bears an aldehyde on one side and an alcohol group on the other. These are expected to join nearby sugars in acetal and ether linkages, respectively. The unsubstituted positions of these furan rings are also available for cross-linking to methylene groups, as in the ribose polymer. However, in the case of the fructose polymer, with the 2,5-furan positions both already substituted, only the 3,4-furan carbons are available for attack by carbocations. That attack at these positions indeed occurs, is indicated by the joint survival of 3,4-furan and methylene signals in DQF spectra of polymers prepared from [3-¹³C]-D-fructose and [4-¹³C]-D-fructose (Fig. 5). The fact that the 3,4-furan signals in the DQF spectrum correspond to the downfield shoulder of the peak in the cross-polarization (CP) spectrum is consistent with the reduced shielding that is generally observed upon substitution.

CONCLUSIONS

Oxalic acid supports the polymerization of short sugars as effectively as ammonium acetate, other organic salts of amines, or amino acids. The absence of pyrroles in the products simplifies the downfield portion of the ¹³C ssNMR spectrum, allowing a more thorough structural analysis. The results show furan units that act to cross-link sugar molecules, rather than form homopolymers. The details of the formation of the furan units and the cross-linking vary between ribose, deoxy-ribose and fructose, although the resulting structures are similar. These findings suggest the complexity that can be found in polymers formed even by homogeneous precursors.

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Figure 1.

Conventional CP (solid lines) and ^1H -dephased (dotted lines) ^{13}C MAS spectra of polymers prepared from unlabeled 2-deoxy-D-ribose (top), D-ribose (middle), and D-fructose (bottom). For optimal visualization, the CP spectra are scaled to have the same amplitude for the most intense peak and the ^1H -dephased spectra are scaled to match the ketone amplitude of the corresponding CP spectrum. The spectrum obtained for the fructose product is very similar to that reported for small “carbon spheres” prepared from aqueous fructose under pressure at higher temperatures.¹⁷

Figure 2.

^{13}C CP MAS spectra of polymers prepared from $[1-^{13}\text{C}]\text{-2-deoxy-D-ribose}$ (top), $[1-^{13}\text{C}]\text{-D-ribose}$ (middle), and $[2-^{13}\text{C}]\text{-D-fructose}$ (bottom).

Figure 3.
 ^{13}C CP MAS spectra of polymers prepared from $[2-^{13}\text{C}]$ -ribose (top) and $[5-^{13}\text{C}]$ -ribose (bottom).

Figure 4. Conventional CP (top) and DQF (bottom) ^{13}C MAS spectra of polymers prepared from $[5-^{13}\text{C}]\text{-D-ribose}$. For optimal visualization, the spectra have been scaled to the same amplitude for the most intense peak.

Figure 5. Conventional CP (solid line) and DQF (dotted line) ^{13}C MAS spectra of polymers prepared from $[3\text{-}^{13}\text{C}]\text{-D-fructose}$ (top pair) and $[4\text{-}^{13}\text{C}]\text{-D-fructose}$ (bottom pair). For optimal visualization, the spectra have been scaled to the same amplitude for the most intense peak.

Scheme 1.

Steps to furans from deoxyribose (top), ribose (middle), and fructose (bottom). For deoxyribose and fructose, simple dehydration of the furanose is consistent with the NMR spectra. For ribose, preserving C1 as aldehyde and committing C2 and C5 as neighbors of the furan oxygen involves dehydration to form dicarbonyl before ring closure.

Scheme 2.

Formation of acetal linkage. In addition to the color scheme used in Scheme 1, carbons depicted in orange represent various carbons from another sugar molecule. The dashed line indicates potential for five- or six-membered ring depending on the relative orientations of the hydroxyl groups.

Scheme 3.

Formation of a furan-methylene linkage. In addition to the color scheme used in Scheme 1, carbons depicted in orange represent various carbons of another sugar molecule.

Scheme 4.

Proposed cross-linking of sugar molecules in the polymeric product from ribose. In addition to the color scheme used in Scheme 1, carbons depicted in orange represent various carbons (2–5) from other ribose molecules.

Chart 1.

Previously proposed structures for melanoidin polymers.⁵ X = NR or O.

Table 1

The reactivity of pentoses and hexoses with oxalic acid at 65°C and the reported equilibrium populations of the isomeric forms of these sugars in aqueous solution.^{13–15}

Sugar	First appearance of dark product	Pyranose form (%)	Furanose form (%)	Acyclic form (%)
D-ribose	4 hours	80.0	20.0	0.05
2-deoxy-D-ribose	4 hours	77.0	23.0	Not Available
D-fructose	5 hours	67.5	31.5	0.8
D-mannose	4 days	94.0	6.0	0.02
D-glucose	12 days	100	0.14	0.02
D-galactose	46 days	99.1	0.9	0.005