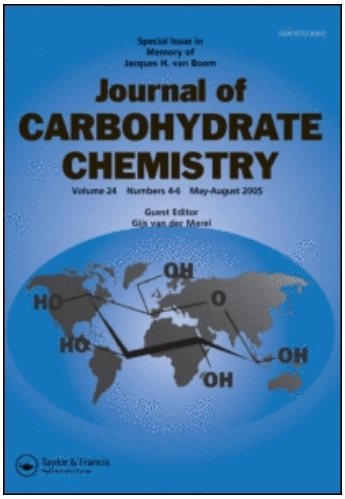

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REACTION OF XYLOSE AND GLYCINE: IDENTIFICATION OF THE MAJOR WATER SOLUBLE COMPONENT

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ABSTRACT

The reaction of equimolar amounts of D-xylose and glycine in D_2O at 68 °C resulted in the formation of a twelve carbon compound, as a major product. No intermediate was detected in the formation of this dimer. The eneaminol, formed on reaction of 0.1 molar D-xylose and glycine in H_2O, after six weeks was isolated and purified. Based on ^1H and ^{13}C NMR, spin echo Fourier transform experiments, and other spectroscopic techniques including FAB-MS and CI-MS of TMS derivative, the structure: N, N[1-deoxy-D-threo-pent-2-enitol,1'-deoxy-β-D-threo-pentose (2',5')] glycine, was assigned to the product.

INTRODUCTION

In 1959, Anet[1] reported the isolation of both mono and diketose amine in the reaction of D-xylose and glycine. These products were not fully characterized, unlike those of D-glucose and glycine. To complement our work[2,3] on the structural

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elucidation of the high molecular weight melanoidins produced in the reaction of D-xylose and glycine, the result of a study of the low molecular weight components of the reaction is now reported. The lack of complete understanding of the reaction mechanism,[4] and the known instability of the diketose compound[1,5] has led us to use ^{13}C NMR as a primary tool for this study.

RESULTS AND DISCUSSION

When the reaction of equimolar amounts of D-xylose and glycine, in D_2O at 68°C, was monitored by ^{13}C NMR, only one major product was detected. Although the solution was dark brown after 48 h, the ^{13}C NMR spectrum showed no additional signals besides those of the starting materials. After seven days the presence of a twelve carbon compound 1, could clearly be seen (Fig. 1). The ^{13}C NMR spectrum of 1, exhibited in addition to a carbonyl carbon, a distinctive low field signal in the unsaturated carbon region. The relative amount of this compound increased with time, until after six weeks, it was the only low molecular weight compound present, besides large amounts of glycine, trace amounts of unreacted xylose and minute amounts of aromatic products.

A parallel experiment, with water as solvent, gave, a ^{13}C NMR spectrum similar to the one obtained in the D_2O reaction. Product 1, was isolated from the reaction mixture after solubilization in anhydrous methanol, and purified by gel filtration chromatography. The number of carbons in the molecule suggested the presence of two units (A and B) one derived from xylose and one from glycine. Reduction with sodium borohydride, followed by acetylation for gas chromatographic analysis, led to decomposition. However, trimethylsilylation of

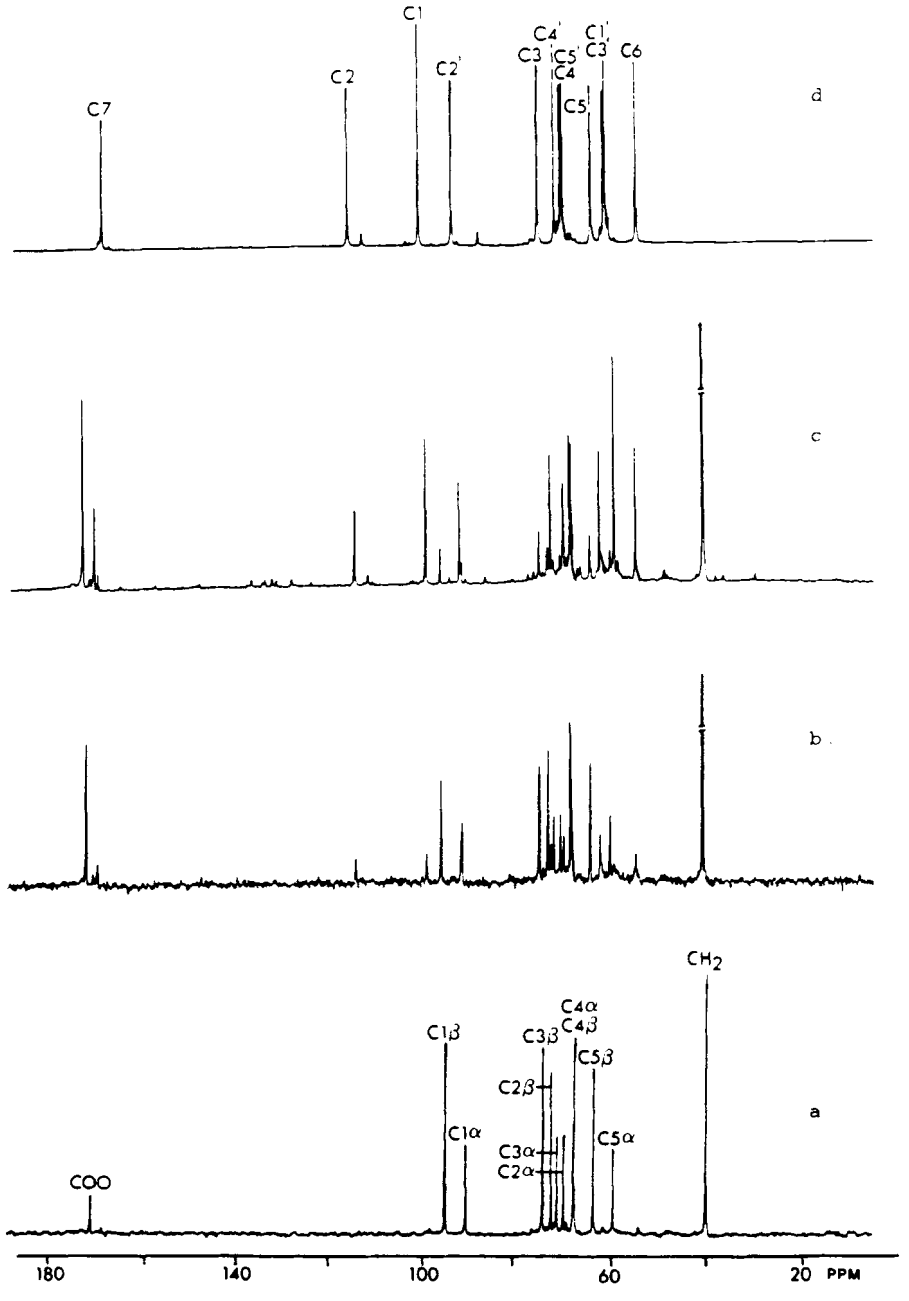


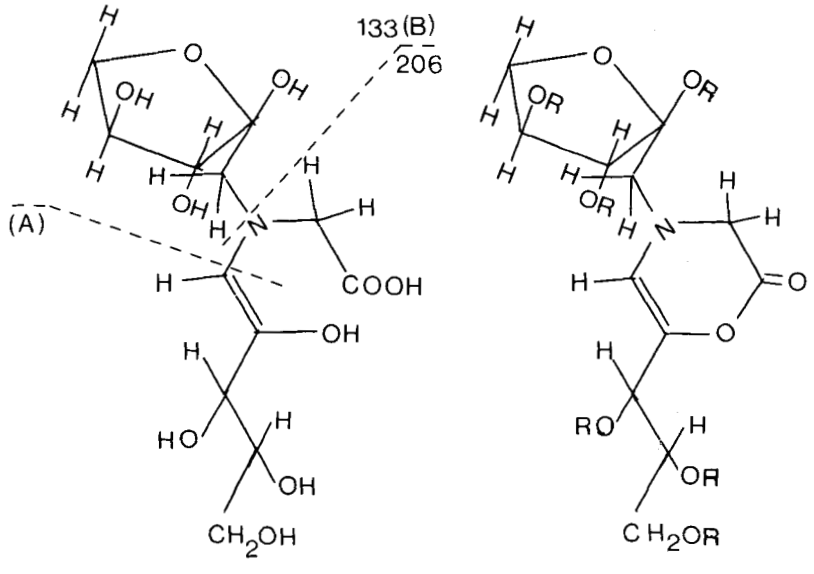
Fig. 1. Reaction of xylose and glycine. Reaction times (days) a, 0; b, 7; c, 30; product, d.

1, followed immediately by gas chromatography on a capillary column, gave only one peak (GC retention time of 1.7 relative to N acetyl muramic acid). The detection of 1 was done using an N/P specific detector. Under the same conditions, neutral sugars could not be detected. The chemical ionization mass spectrum (CI-MS) of this derivative, showed a peak at m/z 754 corresponding to the protonated molecular ion $(M+H)^+$ of the lactone 3. A trimethylsilylated derivative of N-acetylmuramic acid run under the same conditions also gave the protonated molecular ion of the lactone. The fast atom bombardment mass spectrum (FAB-MS) of 1 did not show the pseudomolecular ion $(M+H)^+$ at m/z 340. A linked-scan spectrum[6] of the type B^2/E=constant, however, showed that the peak at m/z 322 arose directly from the unimolecular dissociation of a single parent ion at m/z 340, thus confirming the molecular weight of 1 to be 339, and the nature of the daughter ion at m/z 322 to be $(M+H-H_2O)^+$. This loss of water from the quasimolecular ion of carbohydrates has been reported earlier.[7] Although the peak at m/z 322 could originate from the lactone form of 1, the above linked scan and the presence of the two peaks at m/z 207 (unit A and amino acid moiety) and at m/z 115 (unit B minus H_2O) rules out the latter possibility. From the ^{13}C NMR spectrum in D_2O, it is clear that the product is not in the diketo form since only one signal in the carbonyl region of the spectrum, corresponding to COOH, was observed. The molecule is also nonsymmetrical with respect to the N, as shown by the presence of twelve carbon resonances with different chemical shifts. The large downfield chemical shift of the resonance of the methylene carbon of glycine (55.85 ppm) indicates a tertiary or quaternary amine.[8] The difference of 6 ppm between this chemical shift and that of the methylene carbon of N-(1-deoxy-D-fructos-1-yl)L-glycine(49 ppm)[9] is indicative of a tertiary aliphatic amine,[8] as is the 7 ppm difference

between the chemical shift of the methylene carbon (C-1') of the B moiety (60.3 ppm) and the 53 ppm value reported for the corresponding carbon in fructosyl glycine[9]. The occurence of a resonance at 169.96 ppm, upfield from the reported [9] 171.27 ppm value for the carboxylic acid carbon of fructosyl glycine, is also in agreement with the above data. The ^{1}H coupled ^{13}C NMR spectrum, and the multiplicity sorting data obtained by spin echo Fourier transform experiments[10], showed that the product contains only four methylene, five methine and three quaternary carbons, ruling out a dixylulose glycine as a possible structure. In addition to the quaternary carbon at 169.96 ppm, the molecule contains two other quaternary carbons, one at 115.01 ppm and one at 92.79 ppm. The presence of the 115.01 ppm resonance is strongly indicative of an unsaturated carbon. The possibility of a carbon-nitrogen double bond was eliminated on the basis of chemical shifts assignments,[11] as the carbon would resonate at much lower field. The 115.01 ppm resonance was assigned to an enol carbon, based on the following: (1) the facile enolization of a "diketose amine" at pH 5.5 (pH of the reaction) has been reported;[12] (2) chemical shifts of 117-132 ppm and 99-110 ppm have been reported for =*CH-N< and >N-C=C*< respectively[11]. Introduction of an OH group at *C of the latter functional group, would increase the charge density at that *C, resulting in deshielding of that carbon and shielding of the α carbon; (3) a similar chemical shift has been reported for an enol carbon of ascorbic acid[13]. Although, the 115.01 ppm resonance may also be attributed to an unsaturated carbon of a δ lactone ring, the presence of the 1630 cm^{-1} absorption band in the IR spectrum, eliminates this possibility. Additional evidence for the carbon-carbon double bond came from the ^{1}H NMR spectrum of the HCl salt of 1 in D_2O (a better resolved spectrum than that of the free amine) which showed the presence of only one low

field signal, a singlet at 5.0 ppm attributed to C-1. Although, anomeric protons would occur in this region of the NMR spectrum, the latter chemical shift is also consistent with an ethylenic hydrogen.[14] This is further corroborated by the large coupling constant $J_{C-1,H}$=175 Hz, found in the ^{1}H coupled ^{13}C NMR spectrum of compound 1. When the ^{1}H NMR of 1 was run immediately after dissolution of CD_3OD, the following characteristic signals were observed: a singlet at 15.43 ppm (enol OH), a multiplet at 7.60 ppm (NH^+) and a singlet at 5.12 ppm (ethylenic H). Both IR and UV data support an enol component in the product. The absence in the ^{13}C NMR spectrum of both a keto carbonyl carbon and a second resonance in the unsaturated region, was indicative of a ring form in unit (B) of the molecule. The presence of only twelve resonances in the ^{13}C NMR spectrum showed that the ring form of unit (B), is anomerically pure. The signal at 92.85 ppm, a quaternary carbon, can only be attributed to C-2' of unit (B). The ^{13}C NMR spectrum of the model compound xylulose, showed the presence of two furanose structures α and β, and a keto structure, with the anomeric carbons at 105.73 and 102.97 and the keto carbon at 212.07 ppm. The chemical shifts of the anomeric carbons are very similar to those reported for fructose;[15]; the β fructose structure being predominent. In the case of fructosyl glycine, the C-2 resonance is at 99.0 in contrast to 102.6 ppm in fructose. A second substitution of the H of NH by a second sugar moiety, will lead to an upfield shift of the β carbon, i.e. C-2'. The 92.85 ppm chemical shift is therefore consistent with a β furanose structure. Assignments of the other carbons of the molecule were made by comparison with standard compounds of similar structures.[15,16,17] The ^{13}C NMR spectra of 1 in aqueous solution at pH 2 and neutral pH were the same. However, when a ^{13}C spectrum of 1, was run at a pD of 9, several more resonances appeared in the spectrum. The C-2

resonance at 115.01 ppm and the C-1 resonance at 99.85 ppm were left unchanged, while the C-2 resonance at 92.85 ppm, disappeared and was replaced by two other signals. These results allow us to conclude, that the N atom of the molecule is not involved in the stabilization of the enolic function, as the N would be in the NH^+ form in acid and neutral medium, and in the N form in basic medium. The enolamine, stabilized by hydrogen bonding to the carboxylate anion, as shown on a molecular model, is a more likely form of the molecule. The possibility, however, that the compound exists in the acidic aqueous reaction mixture in the lactone form 2, cannot be excluded; ready cyclization in presence of acid can occur between the COOH and the enol OH groups with elimination of H_2O. Based on the above data, the product was assigned the enolamine structure 1.



1

2 R = H

3 R = TMS

Unlike previously reported data on the reaction of glucose and glycine, this reaction gave only the eneaminol 1. In this

study, no other low molecular weight products were detected in appreciable amount in the reaction. Neither the diketo nor the monoketo amine (simple Amadori compound) isolated by Anet,[1] were seen by ^{13}C NMR or could be isolated from the reaction mixture.

EXPERIMENTAL

General Methods. The ^{13}C and ^{1}H NMR spectra were recorded on a Brucker WM 250 spectrometer. The ^{1}H spectra in D_2O were determined in 5 mm tubes, with SI:16K, SW:3000Hz, pulse delay: 5 sec. with HOD (4.60 ppm) peak as reference. The ^{1}H spectrum in CD_3OD was determined in 5 mm tubes, with SI:16K, SW:5000Hz, pulse delay: 20 sec. with CD_3OH (4.78 ppm) peak as reference. The ^{13}C NMR spectra were recorded in D_2O in 10 mm tubes, SI:16K, SW:15000Hz, pulse delay 10 sec with TMS as external standard. The ^{13}C coupled spectrum was obtained without NOE enhancement. The multiplicity sorting experiment was carried out as reported.[18]

The IR spectrum was obtained using a Beckman 4250 spectrometer. The UV spectrum was obtained on a Bausch and Lomb Spectronic 2000. The Gas Chromatographic analysis was performed on a Hewlett-Packard 5880A GC using a 30 m fused silica capillary column containing a chemically bonded liquid phase (Durabond-1701, J. & W. Scientific, Inc.). Both flame ionization and nitrogen/phosphorous detectors were used with helium as the carrier gas. The GC was interfaced with a 5880A GC terminal. Operating conditions: injection temperature 250 °C, detector temperature 300 °C, column temperature 240 °C, split ratio 80/1, column flow 1 ml/min. The TMS derivative was obtained by treating 2 mg of material in Tri-Sil2 (1 mL) Pierce Chemical Co. Rockford Il. for 20 min at 68 °C. The fast atom bombardment

(FAB) spectrum was obtained on a Finnigan MAT 312 mass spectrometer, interfaced with a Finnigan - Incos 2300 data system. The fast atom beam was produced with a saddle field ion source from Ion Tech, Ltd. (Middlesex, England). The sample was dissolved in glycerol, applied on a copper probe and bombarded with 8 KV xenon atoms at a source pressure of 3-5 x 10^{-5} mbar. The spectrum was obtained at 3KV, resolution 1000 over a mass range of 100-1000, at a scan speed of 3s/decade. The linked-scan spectra were recorded on the same instrument. The unimolecular dissociation reactions occurring in the first-field free region of the instrument were monitored by a linked-scan acquisition where the ratio B^2/E was maintained constant.

For the combined gas liquid chromatography-chemical ionization (isobutane)-mass spectrometry, the TMS derivative was introduced into the mass spectrometer by g.l.c., using an on column injector and a DB-5 Durabond fused silica capillary column (0.32 mm x 30 m), J. and W. Scientific. A temperature programming from 120-240 °C at 15°/min and a helium flow of 2cc/min were used. The mass spectrometer was operated under the following conditions: accelerating voltage 3000 V; ionization energy 250 eV; emission current 0.5 mA; ion source temperature 120 °C. Isobutane was introduced into the source at a pressure of ≈0.7 torr. Thin layer chromatography was carried out on silica gel 60 precoated TLC plates (E. Merck, Darmstadt) and on microcrystalline cellulose plates using n-butanol-acetic acid-ethyl acetate-water (1:1:1:1) and n-butanol-ethanol-water (4:1:5) as solvents respectively. Gel filtration was carried out on Sephadex G-10, Fine, Pharmacia Fine Chemicals. Products were eluted with double distilled water and detected on TLC plates with 30% sulfuric acid in ethanol, silver nitrate-sodium hydroxide, and ninhydrin.

Reaction of D-xylose with glycine. A D_2O solution (2 ml) containing D-xylose (0.300 g) and glycine (0.150 g) was kept at

68°C in a 10 mm NMR tube. A ^{13}C NMR spectrum was run at different time intervals. At the end of six weeks the spectrum showed in addition to large amounts of unreacted glycine and traces of xylose, the presence of one major 12 carbon compound; ^{13}C NMR: δ (ppm) 169.96 (C-7), 115.01 (C-2), 99.85 (C-1), 92.79 (C-2'), 73.65 (C-3), 70.97 (C-4'), 69.68 (C-5'), 69.24 (C-4), 63.32 (C-5), 60.31 (C-1'), 60.29 (C-3'), 55.85 (C-6).

N,N [1-deoxy-D-threo-pent-2-enitol,1'-deoxy-β-D-threo-pentose(2',5')] glycine.1. A solution of D-xylose (15 g) and glycine (7.5 g) in double distilled water (100 mL) was kept under sterile conditions for six weeks at 68 °C. After cooling, the reaction mixture was filtered, the dark brown precipitate was washed with water until the washings were colorless and dried over P_2O_5, yielding a dark brown amorphous material (8.41 g). The filtrate and washings were combined and freeze dried, yielding a light brown hygroscopic substance (6.52 g). A ^{13}C NMR spectrum (D_2O) of the latter, was identical to the one obtained in the D_2O reaction. A portion (0.5 g) of this residue was taken up in anhydrous methanol (50 ml), stirred for 10 min and filtered to remove the insoluble glycine. The filtrate was evaporated under reduced pressure without heating, yielding 0.275 g of methanol soluble product. A portion of the residue, 0.137 g was taken up in double distilled water (1 mL) and chromatographed on a Sephadex G-10 column (75 x 1.5 cm), using water as eluant at a flow rate of 0.64 mL/min and 1 mL fractions were collected. A dark brown substance, subsequently found to be retained in dialysis tubing with a molecular weight cut off of 12,000, eluted in the first 3 mL following the void volume, and weighed 0.008 g after evaporation and drying. The fractions containing the pure desired product were pooled and freeze dried, yielding a light yellow glassy residue which failed to crystallize (0.066 g). An additional 0.020 g could be recovered from the remaining fractions after a second

chromatography. Total recovery of pure product from 0.137 g: 0.086 g, yield: 34% of total water soluble material. The product on TLC silica gel and microcrystalline cellulose gave two spots, probably the enol and diketo forms of the product. On silica gel $Rf/_{xyl}$=0.52; $Rf/_{xyl}$=0.44. On micro crystalline cellulose: $Rf/_{xyl}$=0.58; $Rf/_{xyl}$=0.34. ^{13}C NMR (D_2O), ^{1}H decoupled δ (ppm), with multiplicity sorting results in brackets. 169.96 (C), 115.01 (C), 99.85 (CH), 92.79 (C), 73.65 (CH), 70.97 (CH), 69.98 (CH_2), 69.24 (CH), 63.32 (CH_2), 60.31 (CH_2), 60.29 (CH), 55.85 (CH_2). ^{13}C NMR (D_2O), ^{1}H coupled, δ (ppm): 169.69 (s, COO), 114.85 (s, C-2), 99.61 (d, C-1, 175 Hz), 92.57 (s, C-2'), 74.04 (d, C-3', 157 Hz), 70.55 (d, C-4', 151), 69.35 (t, C-5', 151 Hz), 68.85 (d, C-4, 142 Hz), 62.91 (t, C-5,142 Hz), 60.04 (t, C-1', 151 Hz), 59.86 (d, C-3, 147 Hz), 55.29 (t, C-6, 145 Hz). ^{1}H NMR (D_2O, 250 MHz) δ 5.00 (s,1, H-1), 4.28 (q,1), 4.18 (d,1, 12 Hz), 4.04 (d,1, 17 Hz), 3.90 (m,3), 3.75 (d,1, H-6a, 12.8 Hz), 3.67 (t,1, 14 Hz, 7 Hz), 3.34 (d,1, H-6b, 12.8 Hz), 3.24 (q,3) partial ^{1}H NMR (CD_3OD, 250 MHz) δ 15.43 (s, enol-OH), 7.60 (m, NH^+), 5.12 (s,1, H-1). The instability of the isolated and purified eneaminol 1, precluded the use of microanalysis. IR of HCl salt (KBr) cm^{-1} 3360 (OH), 2970 (CH), 2760 (OH, enol), 2520 (NH^+), 1750 (C=O, COOH), 1640 (C=C). IR (KBr) cm^{-1} 3440 (OH), 2970 (CH), 2760 (OH, enol), 2600 (NH^+), 1630 (COO^-), 1400 (COO^-). FAB-MS, m/z 322 (M+1-H_2O), 207 (M+1-133), 115 (M+1-207-H_2O). On GC, the TMS derivative gave one peak: R_T=1.67 R_T muramic acid; CI-MS (TMS derivative): m/z 754 $(M+1)^+$ of lactone. UV (H_2O) λ max 340 (ε=1220).

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