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Research Article

Glycosylation of Aromatic Amines I: Characterization of Reaction Products and Kinetic Scheme

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Abstract. The reactions of aliphatic and aromatic amines with reducing sugars are important in both drug stability and synthesis. The formation of glycosylamines in solution, the first step in the Maillard reaction, does not typically cause browning but results in decreased potency and is hence significant from the aspect of drug instability. The purpose of this research was to present (1) unreported ionic equilibria of model reactant (kynurenine), (2) the analytical methods used to characterize and measure reaction products, (3) the kinetic scheme used to measure reaction rates and (4) relevant properties of various reducing sugars that impact the reaction rate in solution. The methods used to identify the reversible formation of two products from the reaction of kynurenine and monosaccharides included LC mass spectrometry, UV spectroscopy, and 1-D and 2-D ^{1}H–^{1}H COSY NMR spectroscopy. Kinetics was studied using a stability-indicating HPLC method. The results indicated the formation of α and β glycosylamines by a pseudo first-order reversible reaction scheme in the pH range of 1–6. The forward reaction was a function of initial glucose concentration but not the reverse reaction. It was concluded that the reaction kinetics and equilibrium concentrations of the glycosylamines were pH-dependent and also a function of the acyclic content of the reacting glucose isomer.

KEY WORDS: glucose; glycosylamines; glycosylation; kinetics; Maillard reaction; reducing sugar-aromatic amine.

INTRODUCTION

The reactions of aliphatic and aromatic amines with aldehydes to form imines are important in both drug stability and synthesis. The reactions of glycosidic aldehydes with amines involve the added complexity of monosaccharide equilibria in solution whereby the reversible formation of an imine (acyclic sugar moiety) is in equilibrium with the glycosylamine isomers (the reduced cyclic sugars) (1,2). Amadori products may subsequently form and give rise to additional degradation products. The complex so-called Maillard reaction pathways result in potency loss and product discoloration. All reducing sugars have been implicated in these browning reactions including glucose, lactose, galactose, mannose, and dextrates (3). Glucose is commonly used as a solute in solutions for parenteral administration; lactose is used as an excipient in both parenteral and enteric formulations.

Examples of drug instability due to the Maillard reaction include both primary and secondary amines as in the case of amphetamines, insulin, fluoxetine, sulfa drugs, procainamide, metoclopropamide, and daptomycin to name a few (4–9). For example, Duval *et al.* studied the browning of dextroamphetamine sulfate solutions containing lactose and found that the solution darkens on storage at 50°C with the formation of brownish precipitate (4). Using infrared data and thin-layer chromatography, they concluded that the product was a Schiff base formed from the reaction of the primary amine in dextroamphetamine and the carbonyl group of lactose. Secondary amines in generic formulations of fluoxetine HCl containing lactose as a common ingredient were shown to be less stable than the formulations containing starch due to the Maillard reaction between the drug and lactose (5). Dextrates obtained by the controlled hydrolysis of starch used as excipients are also known to exhibit the browning reaction due to the presence of varying amounts of dextrose (4,10,11).

The majority of previous studies primarily focused on reactions of glucose with model aliphatic amines such as amino acids (12–16) and the browning process due to the formation of Amadori products and advanced Maillard reaction products (15,17–21). However, the formation of glycosylamines in solution, the first step in the Maillard reaction, does not typically cause browning but results in decreased potency and is hence significant from the aspect of drug instability. The glycosylamines of cyclic sugars can exist in equilibria in solution predominantly as the α and β forms

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similar to the parent sugar though the equilibrium compositions may differ. The α, β-glycosylamines mutarotate and hydrolyze through the acyclic imine (22,23).

In the area of drug stability, only a few cases have been reported for reactions of weakly basic aromatic amine-containing drugs in the presence of reducing sugars like glucose forming glycosylamines. For example, the reversible formation of glycosylamines for procainamide in the presence of 5% dextrose solution was reported in water (24,25). In another study, a 20–30% procainamide potency loss (aromatic amine pK_a=2.75) in 24 h was reported following admixing with glucose solutions (26). The formation of glucosylamines has also been reported for the oral suspension of sulfamethoxazole (aromatic amine pK_a=1.69) in the presence of glucose and a limit for the amount of glycosylamines allowed in the suspension has been reported for such formulations (27). In another study involving dissolution testing of Bactrim DS® tablets (sulphamethoxazole, 800 mg and trimethoprim, 160 mg) in the presence of 5% glucose, glucosylamines readily formed with the sulphamethoxazole and suggested the potential of reduced bioavailability of sulphamethoxazole in the presence of dietary glucose (7). Lucida *et al.* studied the reaction of glucose and sulphamethoxazole and showed a pH and temperature-dependent rate of reversible formation of glycosylamines in the acidic to neutral pH range of 1–6 (6). Thus, the kinetics of formation of glycosylamines with respect to solution conditions such as pH, buffers and temperature, effect of aldehydic content of sugar, and an understanding of the mechanism of their formation is a key factor in predicting reactivity and degradation kinetics of these sugar amine reactions. A systematic study involving formation and characterization of the glycosylamines and a detailed kinetic and mechanistic understanding of their formation for weakly basic aromatic amines and sugar carbonyls has not been reported.

Daptomycin, a cyclic lipopeptide antibiotic, has been reported to react with 5% dextrose and other reducing sugars to form reversible products that decrease the potency of the drug in acidic solutions (28). The peptide portion of daptomycin consists of 13 amino acid residues connected at the N-terminal tryptophan to a decanoyl aliphatic group. It has six ionizable groups: four carboxylic acid side chains and two primary amines from the side chains of ornithine and a weakly basic amine kynurenine. The pK_a of kynurenine in daptomycin was determined to be 0.8 using UV spectroscopy (29). Reactions of daptomycin with glyceraldehyde resulting in the formation of Schiff's base are also reported (9). Reaction products formed with dextrose were proposed to be the glycosylamines formed at the kynurenine aromatic amine by reaction with the aldehydic glucose.

This paper represents the first in a series of papers describing the kinetics and mechanisms of the reactions of reducing sugars and weakly basic amines in aqueous solution, solid-state, and pharmaceutical formulations. The main objectives of this first paper is to present (1) unreported ionic equilibria of model reactant (kynurenine), (2) the analytical methods used to characterize and measure reaction products, (3) the kinetic scheme used to measure reaction rates, and (4) relevant properties of various reducing sugars that impact the reaction rate in solution.

Kynurenine is a weakly basic aromatic amino acid with three ionizable groups *viz*. an aromatic amine, an alpha carboxylic acid, and an alpha amine (Fig. 1) with a molecular weight of 208.1. Glucose is one of the most common reducing sugars (MW=180) that exists in solution as cyclic six-membered rings as α or β glucose which equilibrates through an acyclic aldehyde by mutarotation. The α and β glucose differ in the position of the anomeric proton (proton at the C_1 position in the ring) being in the equatorial or the axial positions**,** respectively. The acyclic form of the sugar is the reactive aldehyde in carbonyl amine reactions. All reducing sugars can undergo reactions with amines owing to the aldehydic form. Thus, isomers of glucose like galactose, mannose, gulose, and allose with varying proportion of the acyclic form can all undergo reactions with amines (30). Although the six-membered ring forms are the more stable thermodynamically, glucose also exists as five-membered ring forms in very low concentrations (30). The reaction between kynurenine and glucose (Fig. 2) gives rise to the imine that exists in equilibrium with the cyclic α, β-glycosylamines.

Reactions were initiated with kynurenine and these monosaccharides to isolate the reaction products and identify them. A reaction scheme was proposed based on observed kinetics for these sugar amine reactions. The aromatic amine pK_a for kynurenine was determined using UV spectroscopy; the α-amine acid pK_a was determined using potentiometric titration and the α-carboxylic acid pK_a was determined using 2D-HMQC nuclear magnetic resonance (NMR) spectroscopy. Reaction products were generated by heat-stressing mixtures of kynurenine in the presence of excess sugars under acidic conditions at various pH values. Separation of the reaction products was achieved by reversed phase-high-performance liquid chromatography (RP-HPLC) and product characterization was done by liquid chromatography-mass spectrometry (LC-MS). For reactions of kynurenine with glucose, additional characterization studies were conducted by collecting fractions of products which were lyophilized and evaluated by UV spectroscopy, 1-D ^{1}H NMR, 2-D ^{1}H–^{1}H COSY, and decoupling NMR experiments.

After product identification, a reaction scheme was proposed and confirmed by determining the effect of varying concentrations of glucose on the apparent pseudo first-order loss of kynurenine. The effect of pH on the extent and half-life of the reaction was determined in acidic aqueous solutions (pH values between 1 and 6). The effect of varying acyclic content of various sugars on reactivity was also studied.

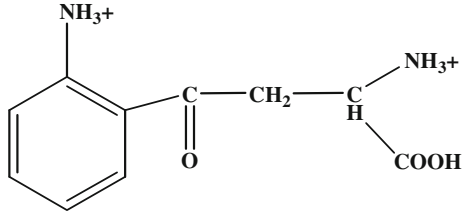

Fig. 1. Structure of kynurenine

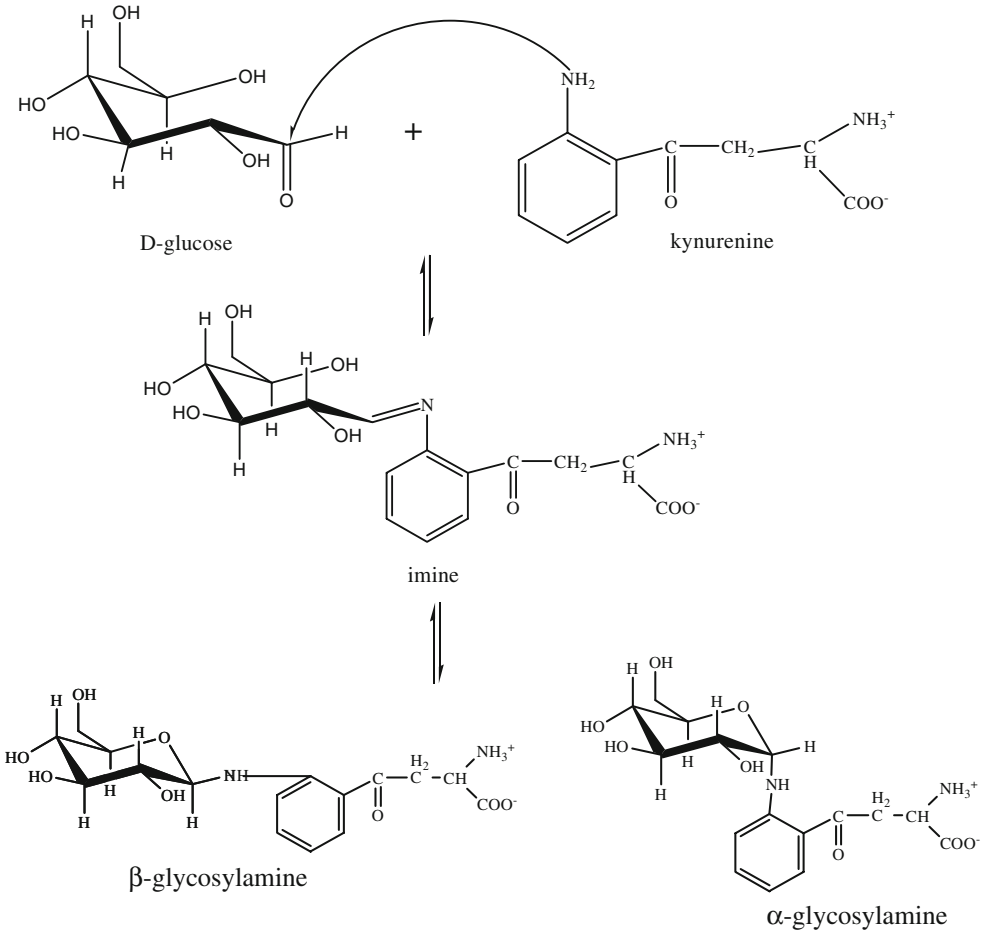

Fig. 2. Reaction of kynurenine and glucose forming imine and α and βglycosylamines

METHODS AND MATERIALS

Kynurenine was obtained from ICN Biomedicals. Anhydrous glucose was obtained from Fisher (Springfield, NJ, USA) as was hydrochloric acid (1 N), sodium hydroxide (0.1 N), and sodium chloride. D-allose was obtained from TCI America; D-gulose was obtained from O-Micron Biomedicals, Inc.; and D-Mannose and D-galactose were obtained from Sigma Aldrich. All chemicals were reagent grade. Solvents used for chromatography were HPLC grade.

pK_a Determinations

The pK_a value of the aromatic amine was determined using a UV spectrophotometric method (Hewlett Packard HP 8453) at 40±1°C (31). A 5.07 mM of L-kynurenine stock solution was used to prepare a series of 0.507 mM solutions of kynurenine in the pH range of 0.46–3.58 with buffers (hydrochloric acid, acetate buffer, and phosphate buffer) and sodium chloride (to adjust to a constant ionic strength of μ=0.5). The absorbance at 281 nm was the wavelength of maximum difference between the ionized and unionized species and was used to estimate the apparent pK_a of the aromatic amine by non-linear regression (JMP®) using an equation relating the absorbance to the fraction and absorptivity of each species (31).

The pK_a value for the alpha amine group on the amino acid side chain was determined using potentiometric titration (DL25, Mettler Inc.). All measurements were carried out at 40±1°C for a 0.01 M L-kynurenine solution. The pK_a was determined using the Gran plot method.

The alpha carboxylic acid pK_a was determined using NMR spectroscopy (heteronuclear multiple quantum correlation spectroscopy) by following changes in chemical shifts of the alpha carbon proton and beta carbon protons on the side chain of kynurenine as a function of pH. The HMQC experiment monitors protons attached to only the carbon skeleton of the molecule, specifically by one bond in the version used.

Kynurenine stock solution (0.020 M) was prepared in deuterated water. The pH was measured at 40°C. The pH electrode was equilibrated and calibrated using pH 7 and pH 4 standard buffer solutions also equilibrated at 40±1°C. Kynurenine solution was pipetted into a water-jacketed vessel, equilibrated to 40±1°C and titrated using standardized 1.0 N HCl added in aliquots of 0.010 mL using an automatic titrator (DL25, Mettler Inc.). One millimolar sodium 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) was also added to the solution for referencing the NMR spectra. During the titration, 0.65 mL of kynurenine solution was removed at the

desired pH and subjected to a 1D proton and a 2D HMQC NMR experiment using a 500 MHz NMR, Varian Inc., spectrometer at 40±1°C. The parameters for the 1-D experiment were: averaged number of transients (nt)=8, spectral width (sw)=6,000 Hz, number of points (np)=6,000 points processed with 0.2 Hz line broadening (lb) and zero filled to 128 k points, and a recycle delay of 5 s (d1) in D_2O. For the 2-D HMQC experiment the parameters were as follows: sw1=30,000, ni=128 s phase 1, 30,000/125.659= 12 ppm in proton. The spectra for the HMQC experiments were directly referenced to DSS at high pH and indirectly referenced at low pH.

After obtaining the NMR spectra, the sample was reintroduced into the titration vessel and the titration was continued until the next desired pH value was obtained. The proton on the α chiral carbon and the protons on the β carbon in the side chain were monitored for changes in chemical shifts as a function of the deprotonation of the alpha carboxylic acid group. Plots of chemical shifts *versus* pD for each of the two groups of protons were sigmoidal in nature. pK_a was estimated using non-linear regression (JMP V5.0.1, SAS Institute) using the following equation: (32,33)

$$\delta_{obs} = \delta_{acid}\left(\frac{10^{-pH}}{10^{-pH} + 10^{-pKa}}\right) + \delta_{base}\left(\frac{10^{-pKa}}{10^{-pH} + 10^{-pKa}}\right) \quad (1)$$

where δ_{obs} is the observed chemical shift, δ_{acid} is the chemical shift of the most acidic species (pH* 1.02) and δ_{base} is the chemical shift of the most basic species (pH* 3.75) (pH* denotes the uncorrected pH in deuterium oxide).

HPLC Analysis

HPLC analyses were performed using a Shimadzu RP-HPLC system consisting of an SCL-10AVP system controller, LC-10ATVP pumps, SIL-10ADVP auto-injector, SPD-10AVP UV-VIS detector, CTO-10ASVP column oven, and a FRC 10A fraction collector. Chromatograms were integrated and data stored using Class VP Chromatography Data System software (Version 4.2). The column used was a Phenomenex HydroRP-18, 4.6×250 mm, 4 μ column.

For reactions of kynurenine and glucose, an isocratic method with a mobile phase composition of 1% methanol and 99% water, a flow rate of 1 mL/min, injection volume of 10 μlt, run time of 35 min, sample temperature of 4°C, column temperature of 25°C, and detection wavelength of 257 nm was used. The HPLC method was validated using standard compendial methods. Calibration curves for kynurenine in the range of 0.2 mM to 1 mM were used for calculating concentrations. The precision of the analytical method was determined by multiple analyses of 0.50 mM solutions of kynurenine. The coefficient of variation was determined to be 3.6%. The accuracy of the method was determined by estimating the percent recovery for 44 kynurenine test samples of known concentrations in the range 0.2 to 1.0 mM. The mean, range, and CV for the test set were 98.9%, 91–110%, and 4.8%, respectively. This method was used for identification of products for kynurenine glucose mixtures using LC-MS and all further kinetic analyses.

For separation and LC-MS analysis of reaction products of kynurenine with galactose, mannose, gulose, and allose the column temperature and run time for the HPLC method was further modified to range from 25–40°C and 35 to 50 min**,** respectively

Reaction Product Formation and Isolation

For reactions of kynurenine with glucose, a reaction mixture of 1.2 mM kynurenine and 0.5 M glucose in hydrochloric acid (pH 3.45) was prepared and stored at 40±1°C in a Teflon-coated rubber-stoppered glass vial. Aliquots of reaction mixture were removed and diluted in acetate buffer (0.5 M, pH 5.8) to quench the reaction. Reactions of 1.2 mM kynurenine with 0.5 M glucose solution were conducted in 5.00×10^{-4} N HCl at 40°C. A semi-preparative Shimadzu RP-HPLC system consisting of a SCL-10A system controller, two LC-10ATVP pumps, and SPD-10A UV-VIS detector was used. Separation of reactants and products were obtained using a Phenomenex Synergi semi prep C18 column, 250× 21.2 mm using a 1% methanol–water mobile phase, at a flow rate of 7 mL/min, detection wavelength of 257 nm and a run time of 80 min. Two products **(**I and II) were formed. Products were collected over dry ice, transferred to a bench lyophilizer (Virtis lyocentre) and lyophilized under vacuum until the product appeared to be completely dry upon which they were removed from the lyophilizer, sealed in plastic tubes and stored at −20°C until use. The integrity of the lyophilized products was reconfirmed by HPLC and the products were used for identification by UV and NMR spectroscopy.

Reaction Product Identification and Characterization

Reaction products were identified using UV, NMR, and mass spectrometry. Reaction mixtures of 1.2 mM kynurenine and 0.5 M monosaccharides (glucose, mannose, galactose, gulose, and allose) were subjected to a quadrupole ion trap type mass spectrometer (Thermo Finnigan, San Jose, CA, USA), with a LCQ Deca Ion trap MS, a Surveyor LC, and PDA detector. A positive electrospray type ESI ionization method was used. The sheath gas and auxiliary gas flow rates were 40 and 10**,** respectively. Nitrogen was used as the sheath and the auxiliary gas. The capillary temperature was 350°C and the capillary voltage was 3 V.

The isolated, lyophilized products from reactions of kynurenine with glucose were dissolved in water and scanned by UV spectroscopy from 190–450 nm using a UV spectrophotometer (Hewlett Packard HP 8453 Diode Array) to detect any observable spectral changes between the reactant (kynurenine) and the products.

NMR spectroscopy was conducted on kynurenine and the lyophilized products of kynurenine glucose reactions to determine their structure. Kynurenine and each isolated lyophilized product (I and II) were dissolved in 0.8 mL of deuterated solvent dimethylsulfoxide (DMSO). A 0.65 mL aliquot of this solution was introduced into the NMR tube and subjected to 1-D ^1H NMR experiments using a 500 MHz NMR (Varian, Inc.) spectrometer. Kynurenine and product I was also subjected to 2-D ^1H–^1H COSY NMR experiments, but not product II due to its low concentration. Decoupling

Table I. Experimental Conditions to Determine the Scheme for the Reactions of 1.2 mM Kynurenine and 0.5 M Glucose at 40°C and Ionic Strength of 0.1–0.5 in Hydrochloric Acid or Phosphate Buffer

Buffer	Total concentration M×10^3	NaCl (M)	Glucose (M)	Kynurenine (mM)	pH
HCl	30.0	0.0902	0.501	1.20	1.66
HCl	10.0	0.0891	0.505	1.25	2.11
HCl	0.100	0.100	0.514	1.25	4.39
NaH_2PO_4/Na_2HPO_4	0.121	0.032	0.508	1.19	5.94

experiments were also performed on products I and II setting the decoupler frequency at the frequency of the aromatic amine proton of kynurenine. This simplified the splitting pattern of the protons on the anomeric carbon for products I and II. The coupling constants for the simplified decoupled doublets were calculated for the two products and compared to literature values for the α/β form of glucose to confirm the identity of the α/β isomers. The NMR parameters for the 1-D ^{1}H NMR experiments for determination of kynurenine structure were as follows; np=60,000, number of transients=4, spectral width=6,000, d1=5 s, temperature=25.4, and lb=0.2. Similarly parameters for 2-D ^{1}H–^{1}H COSY experiment for kynurenine were as follows: nt=4, sw=60,000, np=2,048, lb=not used, fn=2,048, temperature=25.4, and d1=4. For the 2-D acquisition sw1=6,000 and ni=512 in seconds. Similarly, parameters for 1-D ^{1}H NMR determination for product I were, np=60,000, nt=16, sw=6,000, d1=10 s, temperature=25.4, lb=0.2, and fn=131,072. The parameters for 2-D ^{1}H–^{1}H COSY experiment were nt=4, sw=6,000, np=4,096 (direct dimension) lb=not used, fn=2,048, temperature=25.4, and d1=7. For the 2-D acquisition sw1=6,000 and ni=1,024 in seconds (indirect dimension). For the decoupling experiment for product I the peak at 9 ppm was decoupled and the following parameters were used: nt=8, sw=6,000, lb=0.2, fn=131,072, temperature=25°C, and d1=10 s. The parameters for 1-D ^{1}H spectral determination for product II were nt=256, sw=6,000, d1=10 s, temperature=25.4, lb=0.25, and fn=131,072. For the decoupling experiment for product II the peak at 9.01 ppm was decoupled and the following parameters were used: nt=256, sw=6,000, lb=0.25, fn=131,072, temperature=25°C, and d1=10 s.

Kinetics of Kynurenine and Reducing Monosaccharides

Reaction mixtures of 1.2 mM kynurenine and 0.50 M glucose in hydrochloric acid or phosphate buffer at the desired pH and ionic strength of 0.10 or 0.50 were stored at 40±1°C in Teflon-coated rubber-stoppered glass vials. The reactions were carried out in the pH range of 1–6. The solution conditions are given in Table I. Aliquots were removed from reaction mixtures at appropriate times quenched with acetic acid/sodium acetate quench buffer (0.5 M at pH 5.8) and stored at 4°C in auto-injector and analyzed by HPLC. Standard calibration curves were used for calculating the concentration of kynurenine and the reaction products in the reaction mixtures. The concentration time profiles for the loss of kynurenine and the appearance of glycosylamines were constructed and reaction extent and half-lives were measured as function of pH.

Reactions were also conducted with 1 mM kynurenine and various initial concentrations of glucose ranging from 0.19–0.70 M in sodium acetate/acetic acid buffer (total buffer concentration 0.279 M) at pH 3.4 and a temperature of 40°C. The reaction conditions are described in Table II.

To study the kinetics with other glucose isomers, reaction mixtures of 1.2 mM kynurenine and 0.5 M of monosaccharides (galactose, mannose, allose, and gulose) in hydrochloric acid (pH 2.7) at an ionic strength of 0.1 were stored at 40±1°C in Teflon-coated rubber-stoppered glass vials. Aliquots of the reaction mixture were removed, diluted with acetate quench buffer, and HPLC analyses were performed. The extent of reaction and reaction half-life as a function of the acyclic content were determined.

Table II. Experimental Conditions of Reactions of 1 mM Kynurenine with Varying Concentration of Glucose in the Range of 0.19–0.70 M in Acetic Acid/Sodium Acetate Buffer at pH 3.4 at 40°C

No.	Glucose (M)	Kynurenine (mM)	pH
1	0.193	1.05	3.49
2	0.323	1.06	3.50
3	0.504	1.05	3.40
4	0.701	1.05	3.49

RESULTS

pK_a Determinations for Kynurenine

The apparent K_a value of 0.01445 (pK_a=1.84) at 40°C for the aromatic amine was calculated from the UV absorption data at the analytical wavelength of 281 nm and the K_a value of 8.995×10^{-10} (pK_a=9.05) at 40°C of the alpha amino group was determined using potentiometric titration.

The pK_a of the alpha carboxylic acid group was determined using 1-D ^{1}H NMR spectroscopy. The proton on the α chiral carbon and the protons on the β carbon in the side chain were monitored in the 2-D HMQC experiment for changes in chemical shifts as a function of the deprotonation of the terminal carboxylic group.

The two monitored protons showed a characteristic pH-dependent change in chemical shift. The chemical shifts *versus* pD (pH+0.4) for each of the two groups of protons resulted in a sigmoidal curve. The proton on the α chiral carbon gave a pK_a value of 2.57 (Fig. 3a), while the protons on the β carbon in the side chain gave a pK_a value of 2.50 (Fig. 3b). The average carboxylic acid pK_a was estimated to be 2.53 in deuterium oxide or 2.11 in water (as described in the DISCUSSION section).

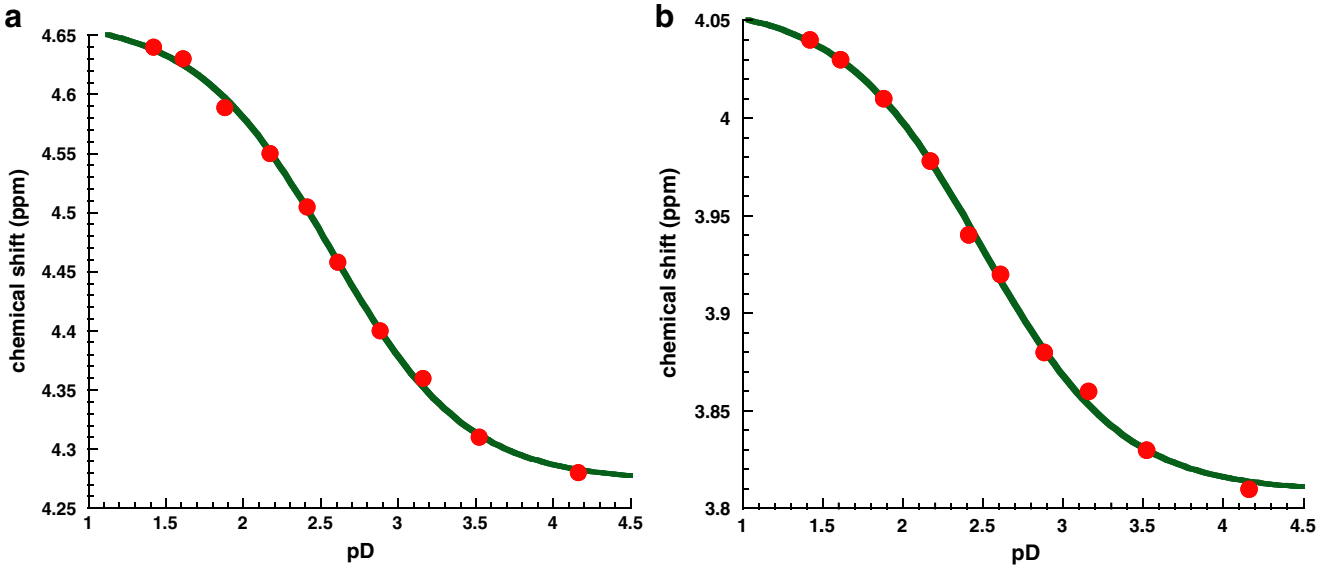

Fig. 3. Chemical shift in the proton frequency for the **a** α chiral carbon proton and **b** β carbon protons as a function of pD in the pH* range of 1.02–3.76. The *solid circles* are the experimental data and the *solid line* was estimated based on non-linear regression using JMP®

Isolation and Characterization of Reaction Products

The HPLC chromatograms for reactions of kynurenine and glucose showed the presence of three peaks; kynurenine and two reaction products; product I and product II in the order of their elution at retention times of 17.3 min, 23 min, and 27.4 min**,** respectively (Fig. 4). The two products were formed reversibly and were isolated for further characterization.

Kynurenine and reaction mixture samples were analyzed using a LC mass spectrometer. The kynurenine sample showed a peak with *m/z* ratio of 209.1, which corresponded to the molecular ion of kynurenine and one proton $(MH)^+$. The first peak in the reaction mixture chromatogram corresponded to the kynurenine molecular ion with *m/z* of 209.1. The two reaction product peaks that eluted from the reaction mixture of kynurenine and glucose corresponded to *m/z* ratio of 371 $(MH)^+$.

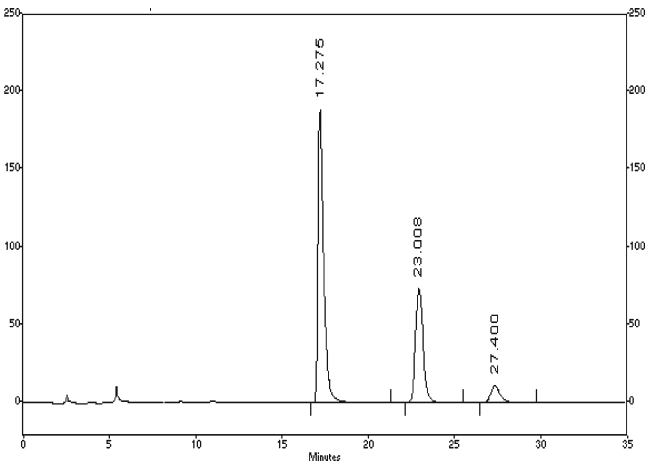

Fig. 4. Representative chromatogram of reaction mixture of kynurenine and glucose peak at 17.3 min is kynurenine, at 23 min is product I and at 27.4 min is product II

Reactions of kynurenine with galactose, mannose, allose, and gulose also gave two major products in addition to the kynurenine peak. In reactions of galactose, the two peaks were not completely separated and co-eluted as a single peak. The LC-MS analysis indicated that the products formed with the other monosaccharides (galactose, mannose, gulose, and allose) also corresponded to a *m/z* ratio of 371 $(MH)^+$ indicating that the same products were formed for reactions of weakly basic amine with all reducing monosaccharides.

The UV scans of the isolated products looked similar to the UV scan of kynurenine exhibiting the same three wavelength maxima of 228–230 nm, 257–260 nm, and 360–362 nm. These results are consistent with the formation of the glycosylamines.

The 1-D proton spectrum of kynurenine, showed two distinct regions of peaks: one at 6–8 ppm with five peaks that corresponded to the aromatic protons and the other region at 3–4 ppm with three peaks that corresponded to the side chain protons. The assignments of the protons for kynurenine were confirmed by performing a 2-D ^1H–^1H COSY experiment (Table III). The COSY spectrum also showed two distinct regions: the aromatic protons in the region of 6–8 ppm and the side chain protons in the region of 3–4 ppm. The 1-D spectra and the 2-D spectrum for product I both showed two regions associated with the aromatic protons at 6–9 ppm and the side chain protons at 3–4 ppm similar to the kynurenine spectra. In addition to these protons, the 1-D spectra for products I and II showed the sugar protons in the region of 3–4 ppm overlapping with the side chain protons of the amino acid. Also, the 1-D spectra showed protons at 4.5 and 5.1 ppm representing the anomeric protons for glucose and protons at 9 and 9.1 ppm representing the aromatic amine protons for products I and II, respectively. The COSY spectrum for product I showed a cross peak between the anomeric proton (4.5 ppm) and the aromatic amine (9 ppm) thereby confirming the identity of the reaction product to be a glycosylamine and not an imine (Fig. 5). The identity of the β and α-glycosylamines was confirmed based on the coupling constants of 7.98 and 3.67

Table III. Chemical Shift Assignments in ppm for Kynurenine Protons for the 2-D ^{1}H–^{1}H COSY Spectrum in Deuterated DMSO

Region	Position	Chemical shift (ppm)
Aromatic region	H1	6.75
	H2	7.25
	H3	6.5
	H4	7.7
	–NH	~7.2
Side chain region	–CH	3.6
	–CH2	3.5, 3.25

determined for the products I and II, respectively, by decoupling experiments conducted for products I and II. The assignments for the glycosylamines are listed in Table IV.

Determination of Reaction Scheme

Reactions of kynurenine in the presence of excess glucose were carried out in the pH range of 1 to 6.5. The kinetic studies were performed under pseudo first-order conditions by maintaining constant pH, temperature, and ionic strength and glucose concentrations in high molar excess. The chromatograms for the reaction mixtures showed the same three peaks at all pH values; kynurenine and the two products indicating that the reaction proceeded to the α and β glycosylamines at all pH values. Typical calibration curves of peak area *versus* concentration for kynurenine and the glycosylamines (concentration range of 0.2–1 mM) were linear (R^2>0.99). The concentration–time profiles (Fig. 6) indicated that kynurenine reversibly formed the two glycosylamines and that equilibrium between all three species was reached over the duration of the reaction. The α and β-glycosylamines equilibrated rapidly with each other and existed as a constant ratio of 6:1(β-glycosylamine–α-glycosylamine) at all pH values. The simplest scheme consistent with the observed kinetics was:

$$\text{kynurenine} \underset{k_{\text{robs}}}{\overset{k_{\text{fobs}}}{\rightleftharpoons}} (\alpha+\beta)\text{glycosylamines} \qquad (2)$$

where k_{fobs} is the rate constant for the forward reaction and k_{robs} is the rate constant for the backward reaction. The

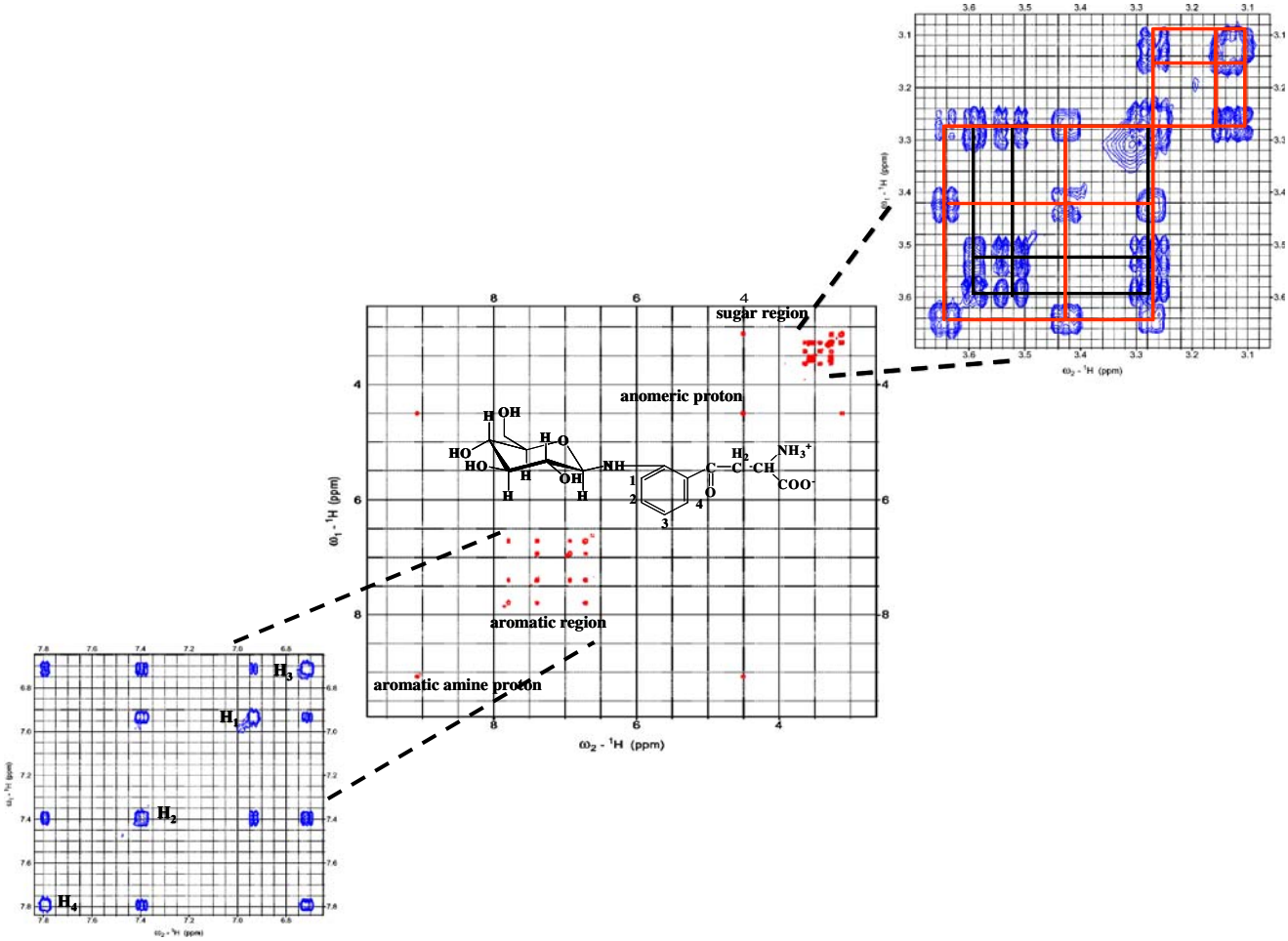

Fig. 5. COSY spectrum of product I showing the region of 3–4 ppm corresponding to overlapped peaks and cross peaks of the side chain and sugar protons and 6–9 ppm corresponding to peaks and cross peaks for the aromatic protons. (*Bottom right*) Expanded region showing the region of 6–7 ppm corresponding to overlapped peaks and cross peaks of the aromatic protons. The peaks at 6.7, 6.9, 7.4, and 7.8 ppm correspond to the aromatic ring protons H_3, H_1, H_2, and H_4, respectively. (*Top left*) Expanded region showing the region of 3–3.7 ppm. Two distinct spin systems corresponding to the aromatic side chain and sugar protons

Table IV. Chemical Shift Assignments for the Glycosylamines Based on 1-D-^{1}H NMR, 2-D NMR in Deuterated DMSO

Region	Position	Chemical shift
Aromatic region		
α form	–NH	9.1
β form	–NH	9.0
	H1	6.9
	H2	7.4
	H3	6.7
	H4	7.8
Side chain region	–CH	3.6
	–CH2	3.5, 3.25
Sugar region		
α form	H1	5.1
β form	H1	4.5
	H2	3.1
	H3	3.16
	H4	3.3
	H5	~3.3
	H6	3.65
	H6'	3.44

observed rate constant (k_{obs}) for the overall loss of kynurenine was determined based on the pseudo first-order reversible kinetics of the reaction. Table V lists the reaction half-lives and equilibrium concentrations of kynurenine for the reactions of kynurenine and glucose in the acidic pH range of 1 to 6.

Reactions were conducted with 1.2 mM kynurenine with various initial concentrations of glucose from 0.19–0.70 M. The data was fit using reversible reaction kinetics and the forward and reverse rate constants were determined using the Eq. 2 (Table VI). Typical CV values for rate constant estimates were 10%.

Reactions of kynurenine with other reducing sugars *viz.* galactose, mannose, gulose, and allose also resulted in the formation of the glycosylamines. The reaction kinetics for these reducing monosaccharides was also described by the reversible reaction scheme shown in Eq. 2. The total acyclic content for the various isomers of glucose were obtained from literature, based on measurements in aqueous solutions in deuterium oxide at 30°C obtained by ^{13}C NMR for these sugars (34). The acyclic content was reported to be lowest for glucose and allose at 0.009% while gulose had the highest at 0.082%. Equilibrium concentrations of kynurenine for reactions with allose, gulose, galactose, and mannose indicated that the equilibrium concentration was constant through all the sugars averaging at 64.3% (±3.4%) of the initial amine concentration. Reaction half-lives indicated the longest half-life for glucose and allose at 0.6 h and 0.5 h, respectively, and the shortest for gulose at 0.06 h.

DISCUSSION

Determination of pK_a Values

Kynurenine is an aromatic amino acid with three ionizable groups; the aromatic amine, the alpha amine, and the alpha carboxylic acid. The aromatic amine pK_a was estimated to be 1.84 at 40°C using UV spectroscopy. Kynurenine is a weakly basic aromatic amine similar in basicity with primary aromatic amine-containing drugs such as sulfamethoxazole (pKa=1.69) (6), procainamide (pKa=2.75) (26), benzocaine (pKa=2.49), or procaine (pKa=2.28) (35) to name a few (31). The alpha amine pK_a value was determined to be 9.05 at 40°C by potentiometric titration. Protonation of the reactive amine dramatically reduces its nucleophilicity thereby substantially minimizing its reactivity. Hence, the alpha amine is largely unreactive in the acidic pH range of 1–6 and the reactions with glucose were limited to those involving the weakly basic aromatic amine. The alpha carboxylic acid pK_a for amino acids typically lies in the range of 2–3. This is very close to the aromatic amine pK_a and hence to avoid interference by the aromatic amine pK_a, an HMQC type NMR experiment was used to determine the pK_a for the carboxylic acid. In the HMQC experiment, protons attached to the carbon skeleton of the molecule were monitored. Since the carboxylic acid proton is exchangeable, the protons on the carbon atoms (α and β carbons of the side chain) neighboring to the carboxylic acid group were monitored as a function of pH. The protonation or deprotonation of the carboxylic acid changed the shielding of the neighboring protons and caused a change in chemical shift of those protons as a function of pH. This chemical shift was plotted as a function of pD to yield a sigmoidal curve that was used to estimate the pK_a value. The standard correction for estimation in deuterium was applied to the observed meter reading (36,37). pK_a value of 2.57 for the α carbon proton and 2.50 for the β carbon protons gave an average pK_a value of 2.53 for the carboxylic acid pK_a at 40°C in D_2O. However, the values for ionization constants of carboxylic acids in deuterium oxide and water differ by a factor of 0.5–0.6 due primary isotope effects (38,39). The difference in the ionization constant (ΔpK_a) of carboxylic acids of amino acids in

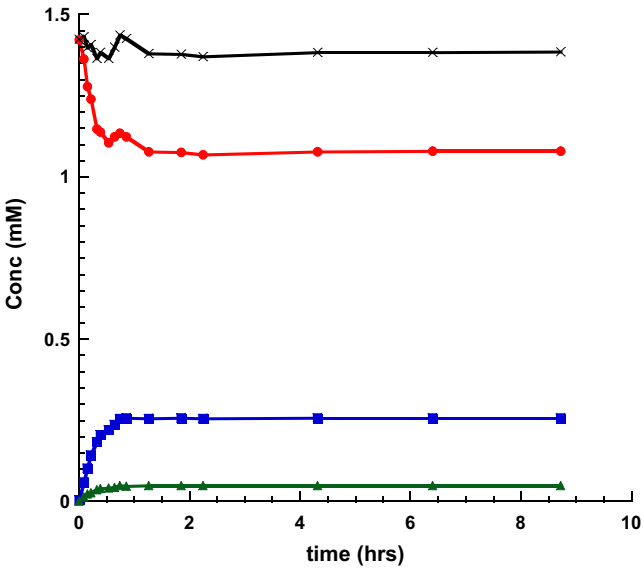

Fig. 6. Typical concentration area time profiles of reaction of 1.2 mM kynurenine (*solid circle*) and 0.5 M glucose in HCl/NaCl at 0.1 ionic strength and 40°C. Appearance of β-glycosylamine (*solid square*), α-glycosylamine (*solid triangle*), and mass balance (*multiplication symbol*) are depicted

Table V. Overall Observed Rate Constant (k_{obs}), Half-Lives (h), and Percent of Initial Concentration of Kynurenine at Equilibrium for the Reactions of 1.2 mM Kynurenine with 0.5 M Glucose in Hydrochloric Acid and Phosphate Buffer at an Ionic Strength of 0.1–0.5 and 40°C in the pH Range of 1 to 6.5

Buffer	pH	k_{obs} (h^{-1})	Half-life (h)	Percent of initial concentration at equilibrium
HCl	1.66	3.87	0.179	78.58
HCl	2.11	2.43	0.285	72.20
HCl	4.39	0.118	5.87	65.24
NaH_2PO_4/Na_2HPO_4	5.94	0.026	26.86	63.27

The estimated rate constant values for the forward and reverse reactions are contained in paper "Glycosylation of aromatic amines II: Kinetics and Mechanisms of the Hydrolytic Reaction between Kynurenine and Glucose" (in press)s

water and deuterium oxide can be described by a linear relation of the form (y=mx+h) as:

$$pK_D - pK_H = \Delta pK = a + b\ pK_H \quad (3)$$

where a=0.332 and b=0.040 (38). Substituting the pK_a value in D_2O (pK_D) of 2.53 for kynurenine in the Eq. 3, the final pK_a value for kynurenine in water (pK_H) was 2.11. This is consistent with the typical pK_a values in the range of 2–3 reported for the alpha carboxylic acids of amino acids (31).

Identification of Reaction Products

In the pH range of 1–6, the aromatic amine of kynurenine is largely unprotonated and therefore present in a reactive form, whereas the aliphatic alpha amine is largely protonated and unreactive. The reaction of kynurenine and glucose gave rise to two products I and II. LC-MS, UV spectroscopy, and NMR were used to determine the structure of the two products. Both the products, I and II, showed a *m/z* of 371 or identical molecular mass of 370 indicating that the products were either the glycosylamines or the imine. The *m/z* of 393 in the mass spectra for the two products corresponds to molecular ion plus sodium. Though the expected products were the glycosylamines, as has been reported for reactions of amines with sugars (6,8,26) this was further confirmed using UV and NMR spectroscopy. The two products showed similar UV spectral properties to that of kynurenine. The presence of an additional double bond in an imine would result in a shift of the UV wavelength maxima to a longer wavelength. Since no such shifts were observed, the two products were most likely the two glycosylamines. This supposition was further confirmed using NMR spectroscopy. 1-D ^1H NMR, 2-D ^1H–^1H COSY experiments and decoupling experiments were conducted to confirm the identity of the products.

Table VI. Estimated Values for the Forward (k_{fobs}) and Reverse Rate (k_{robs}) Constants for Reactions of 1 mM Kynurenine with 0.19–0.70 M Glucose in Acetic Acid/Sodium Acetate Buffer at pH 3.4 and 40°C

Concentration of glucose (M)	k_{fobs} (h^{-1})	k_{robs} (h^{-1})
0.193	0.25	1.84
0.323	0.50	2.56
0.504	0.75	2.29
0.701	1.19	2.77

The chemical shift assignments for the protons on the amino acid kynurenine are listed in Table III. The proton at 3.6 ppm was typical of the α chiral carbon proton and showed a doublet of doublets due to the two nonequivalent protons on the side chain that appeared at a chemical shift of 3.5 and 3.2 ppm. The proton at 7.2 ppm showed a typical broad singlet characteristic of an amine proton. A triplet at 6.5 ppm, a doublet at 6.75 ppm, another triplet at 7.3 ppm, and a doublet at 7.7 ppm each corresponded to one proton on the aromatic ring of kynurenine. In the COSY spectrum, the peaks across the diagonal represent the 1-D NMR peaks. The cross peaks perpendicular to the peaks along the diagonal represented connectivity between corresponding peaks along the diagonal. Protons up to two to three bond distances away typically show cross peaks. Occasionally longer range couplings also show in a COSY. In the aromatic region of 6–8 ppm, cross peaks were observed for four peaks along the diagonal except one peak which was the broad amine peak. The assignments of the aromatic region protons in kynurenine were based on the splitting pattern of peaks in the 1-D spectrum for kynurenine and product I, and the cross peaks in the COSY spectrum. Substituent group rules and resonance effects were also used to verify the assignments in the aromatic region.

The identity of the glycosylamine was determined using the following:

1. The presence of the anomeric proton and aromatic amine proton indicates the presence of an intact sugar ring.
2. A cross peak in the COSY spectrum for product I showed that the anomeric proton and the amine proton are connected in the structure through two to three bonds.
3. Decoupling experiments identified the α and β forms of the glycosylamines.
4. Chemical shifts were assigned to complete the structural identification.

1-D NMR spectrum of product I and II in deuterated DMSO showed a doublet at 9.0 and 9.1 ppm that were assigned to the aromatic amine protons for products I and II, respectively. The triplet at 4.5 ppm for product I and at 5.1 ppm for product II was typical of the anomeric proton on the glucose and was assigned to be the C_1 proton (anomeric proton) on the glucose ring. A 2-D ^1H–^1H COSY experiment was conducted for product I (Fig. 5). It showed a distinct cross peak between the proton at 9.0 ppm and the proton at

4.5 ppm, which indicated that they were two to three bonds away, and hence the two peaks were assigned to be the amine and the anomeric proton, respectively. In order to further confirm these assignments and identify the α and β forms, the decoupling experiments were used that provided two pieces of information:

1. Decoupling of either of those protons affected the splitting pattern of the other proton, indicating the two protons were connected.
2. It could be used to distinguish between the isomeric forms i.e. β/α forms.

The decoupling experiment was performed by setting the decoupler frequency at the frequency of the aromatic amine peak for each of the products I and II. Thus, the effect of the aromatic amine proton on the anomeric proton of glucose was removed and simplifying the splitting pattern. The anomeric proton splitting pattern changed from a triplet to a doublet. This confirmed that the protons at 9.0 and 9.1 ppm and the anomeric protons at 4.5 and 5.1 ppm were connected in the structure for products I and II, respectively. The coupling constant (J) for the decoupled doublets was determined to be 7.98 for product I and 3.67 for product II. These coupling constants were typical to that of the β-glucose (J~8) and the α-glucose (J~4), respectively (6,30,40). The result supported the assumption that product I was the β glycosylamine and the product II was the α glycosylamine. The more stable form, the β glycosylamine, appeared in a higher ratio in the reaction mixture, in agreement with the observations of Sunderland and coworkers for the reaction of glucose and sulfamethoxazole (6).

Figure 5 shows the expanded COSY spectrum in the region of the aromatic protons for product I. The chemical shift assignments were done using the kynurenine assignments as a reference and also considering the splitting patterns of the peaks in the 1-D NMR spectrum. The region of 3–3.7 ppm in the COSY spectrum showed two spin systems

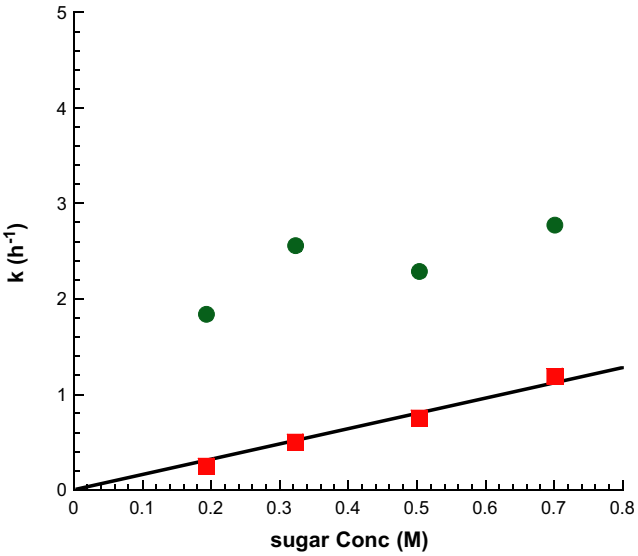

Fig. 7. The forward (*solid circle*) and reverse (*solid square*) rate constants as a function of initial glucose concentration in sodium acetate/acetic acid buffer at pH 3.49 and 40°C

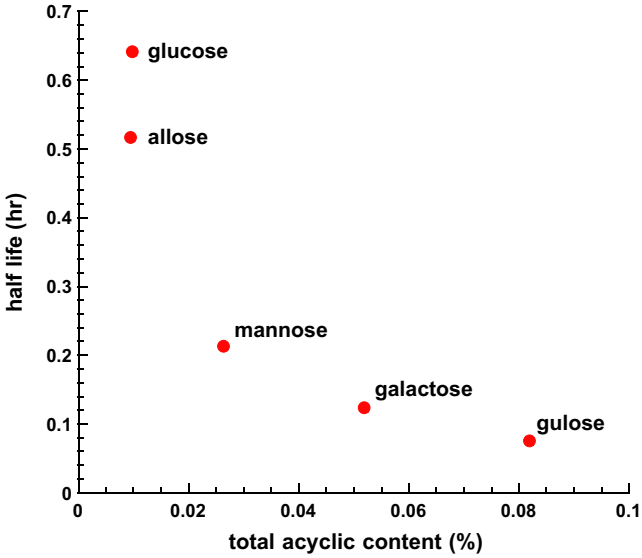

Fig. 8. Half-life (h) *versus* total acyclic content (%) for reactions of 1.2 mM kynurenine with 0.5 M monosaccharides for reactions at pH 2.7, 0.1 ionic strength, and 40°C

overlapping each other, one from the protons in the side chain of the amino acid and the other from the sugar protons (Fig. 5). One of the spin system overlayed with the kynurenine side chain region and was assigned accordingly. The other spin system was due to the sugar protons. Specific proton assignments for the sugar part of the glycosylamine (Product I) was done by initially assigning the anomeric proton (C_1) at 4.5 ppm followed by assigning the proton at 3.1 ppm as C_2 based on the cross peak observed in the COSY spectrum. Similarly the subsequent protons were assigned using cross peaks, peak integrations, and comparing typical assignments reported in literature for a β D-glucose (30). Proton signals for the amino acid side chain and sugar ring which lie around 3.2–3.35 ppm are also overlapped by the water peak due to interference of water signals in this region of the spectrum. The chemical shift assignments for the glycosylamines are reported in Table IV. The chemical shifts for the glycosylamines for the anomeric protons correlated well with those determined by Lucida and co-workers (6). The product II was identified based on the mass, the coupling constant (J), and similar chemical linkages identified for the 1-D spectra of products I and II. However, individual chemical shift assignments for product II were not done because the low concentrations and instability of the sample resulted in a noisy spectrum. The characterization studies demonstrated that for the reactions of kynurenine and glucose, product I formed was the β-glycosylamine and the product II was the α-glycosylamine.

Reaction Scheme and Kinetics

The reaction of kynurenine and glucose reversibly formed glycosylamines in the pH range of 1 to 6. Based on the concentration time profiles, only three species existed in solution in detectable quantities, kynurenine and the two glycosylamines. The glycosylamines equilibrated in solution with each other through the acyclic imine. This is analogous to the α, β-glucose that exists in equilibrium in solution with

the aldehydic form (23). However, no detectable concentrations of the imine were obtained under the experimental conditions. No loss in mass balance was observed. A simple reversible scheme (Eq. 2) was used to describe the data. The pseudo first-order rate constant (k_{obs}) for the loss of kynurenine was determined based on reversible kinetics. The rate of formation of glycosylamines was pH-dependent. For example, the reaction half-life at pH 1.66 was 0.179 h while the half-life at pH 5.94 was 26.86 h (Table V). pH-dependent reaction rates for the reactions of daptomycin with some monosaccharides were also reported earlier by Inman and Kirsch (28). The reaction extent was also a function of pH as shown in Table V.

Reaction scheme confirmation was obtained by conducting a series of reactions with various concentrations of excess glucose. The forward rate constant (k_{fobs}) was determined to be a function of sugar concentration; however, the reverse reaction rate constant (k_{fobs}) was independent of the glucose concentration (Table VI, Fig. 7).

Reactions of kynurenine with the other monosaccharides indicated that the extent of reaction was not a function of acyclic content; however, the reaction half-life was a function of the total acyclic content (Fig. 8). This indicated that the reaction kinetics for these sugar weakly basic amine reactions were a function of the inherent sugar properties and a higher equilibrium acyclic concentration resulted in increased reactivity.

CONCLUSIONS

Kynurenine contains a weakly basic aromatic amine that reacts with the aldehydic moiety of reducing sugars under mildly acidic conditions to form glycosylamines. The reaction occurs reversibly via a steady-state imine intermediate, and the reaction rate is dependent on the concentration of the acyclic form of the sugar. The pH-dependent mechanisms of this reaction are the subject of the second paper in this series.

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