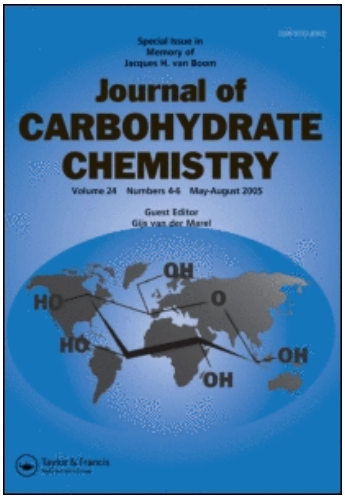


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# High Resolution $^{1}H$ and $^{13}C$ NMR Resonance Assignments, Conformation and Solution Behavior of *N*-Phenyl (*N*-Phenyl-β-D-Glucopyranosylamine) Uronamide in DMSO

P. Irwin; P. Pfeffer; L. Doner; T. Lillie; M. Frey

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# HIGH RESOLUTION $^1H$ AND $^{13}C$ NMR RESONANCE ASSIGNMENTS, CONFORMATION AND SOLUTION BEHAVIOR OF *N*-PHENYL(*N*-PHENYL-β-D-GLUCOPYRANOSYLAMINE)URONAMIDE IN DMSO

P. Irwin,[a*] P. Pfeffer,[a] L. Doner,[a] T. Lillie,[b] and M. Frey[c]

[a]USDA Eastern Regional Research Center
600 E. Mermaid Lane
Philadelphia, Pennsylvania 19118

[b]Department of Chemistry
Lafayette College
Easton, Pennsylvania 18042

[c]JEOL USA, Inc.
11 Dearborn Rd.
Peabody, Massachusetts 01960



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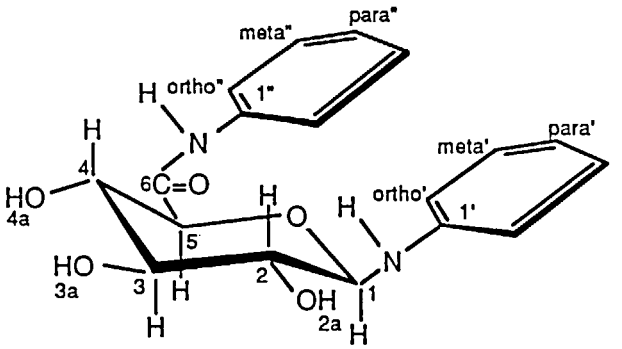
## ABSTRACT

*N*-phenyl(*N*-phenyl-β-D-glucopyranosylamine)uronamide (**1**) and *N*-phenyl(*N*-phenyl-D-galactopyranosylamine)uronamide (**2**) spontaneously formed and precipitated (pH = 4.75 [aq]) in the presence of aniline upon $C_6$ activation with a carbodiimide reagent. In the presence of $H_2O$, **1** and **2** were hydrolyzed to *N*-phenyl-D-glucopyranuronamide (**3**) and *N*-phenyl-D-galactopyranuronamide (**4**), respectively, with a rate constant proportional to the concentration of $H_2O$, with compound **2** degrading 2-4 times faster than **1**. We have unequivocally assigned both the $^1H$ and $^{13}C$ NMR spectra for **1** using a combination of $^1H$-$^1H$ and $^{13}C$-$^1H$ Chemical Shift-Correlation Spectroscopy as well as 2D nuclear Overhauser enhancement (nOe) and natural abundance 2D Double Quantum Transfer experiments. Scalar coupling constants for the directly-bonded pyranose protons of compounds **1** and **2**, 2D nOe data as well as conformational energy minimization calculations indicated the sugar existed almost exclusively in DMSO as the $^4C_1$ conformer.

## INTRODUCTION

Reactions involving the activation of uronic acid carboxyl groups with well-known carbodiimide[1] reagents are biochemically significant since they have been utilized to spin label monosaccharide sugar acids[2] as well as their polymer equivalents.[3] It is also well-known that aldoses can condense with either ammonia or primary amines to form either glycosylamines (*N*-glycosides) via the loss of water, or, in the presence of acid, 1-*amino*-1-deoxyketoses via the Amadori rearrangement.[4] Herein we report that carbodiimide-activated D-glucuronic and galacturonic acids spontaneously formed *N*-phenyl(*N*-phenyl-β-D-glucopyranosylamine)uronamide (compound **1**) and its galacturonic acid equivalent (compound **2**),




1

respectively, at pH = 4.75 (aq) in the presence of aniline. In order to confirm the structure as the title compound, we also have provided spectroscopic evidence that the reaction product's structure and conformation were as depicted above, as opposed to the product that might be derived from the Amadori rearrangement.

## RESULTS AND DISCUSSION

In the process of synthesizing various amide derivatives from plant-derived acid sugars[3] we noted that a white precipitate was formed when D-glucuronic acid was activated with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) at pH = 4.75 (aq) in the presence of aniline. The compound formed

was produced with a yield of ca. 80-85%. Identical reactions with D-galacturonic acid resulted in a similar phenomenon but with a much lower yield (ca. 20-25%). Based upon chemical ionization mass spectrometry data we hypothesized that the white precipitate formed during these reactions was either compound **1** or its Amadori rearrangement product(s) as depicted in Fig 1. Furthermore, we hypothesized that the difference between the D-glucuronic and D-galacturonic acid reactions was related to differences in the reaction rate and solubility in $H_2O$ of the corresponding disubstituted derivatives. This contention was supported by data in Fig. 2 wherein the relative concentrations of **1** and **2** were monitored in various MeOH:$H_2O$ solutions using reverse phase HPLC (Table 1). Under these conditions compounds **1** & **2** were soluble at a low concentration (ca. 1 mM) even in the presence of up to 50% $H_2O$ [v/v]. Compound **2** was hydrolyzed to its amide derivative, **4**, and aniline with a first order rate constant ($k_1$) on the order of 2- to 4-fold larger than the equivalent $k_1$ for **1**; the rate of these hydrolyses was dependent upon the activity of $H_2O$.



We were able to assign a structure to **1** on the basis of standard spectroscopic data.[6] High resolution FT-IR spectra of **1** (KBr pellet) displayed bands at 1664.292 and 1540.539 $cm^{-1}$ which were typical for amide I and II, respectively. We also performed a $^1H$-$^1H$ chemical shift-CQrrelated Spectroscopy (COSY[7]) experiment in DMSO-$d_6$ for **1** (Fig. 3). In this figure the 1D proton spectrum,[8] represented along the diagonal, clearly indicated that two distinct aromatic ring systems existed in this compound based upon off-diagonal connectivities at low field (Fig. 3, dotted lines) which resulted from long-range[9] as well as normal scalar couplings. Unfortunately, because of the quaternary carbons of the phenyl rings (1" and 1') there was not enough information to unequivocally determine which group of aromatic resonances corresponded to the amide or amino substituents. Based upon proton resonance multiplicity, intensity, and chemical shift, it was possible to tentatively assign the 2.86 ppm doublet to

## Reaction Scheme

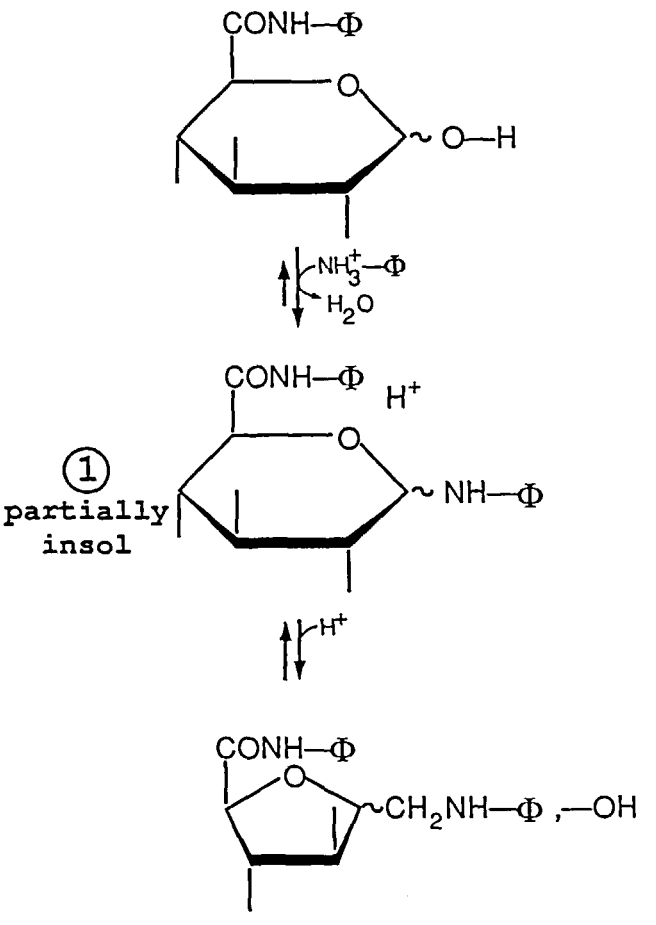



FIG. 1. Reaction scheme[4] proposed for the formation of compound 1 and its anticipated Amadori rearrangement product(s).

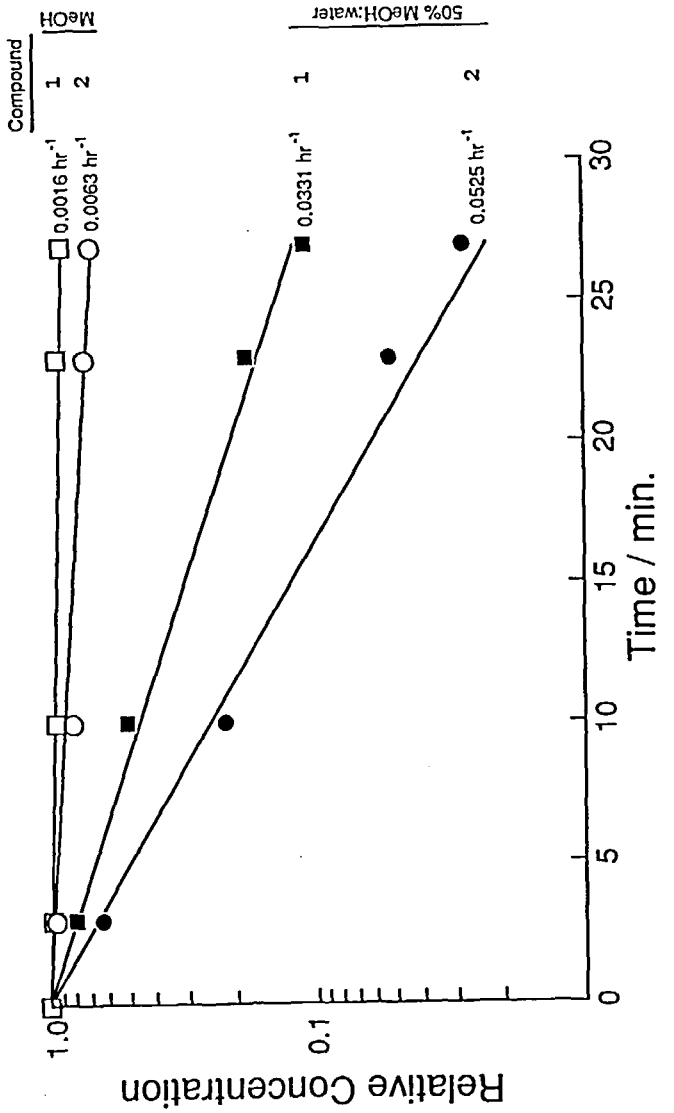



FIG. 2. Change in the relative concentration of compound 1 (squares) and 2 (circles) over time in 100% MeOH (open symbols) and 50% [v/v] MeOH:$H_2O$ (closed symbols) at 40 °C. The abs MeOH solution was not completely dry as evidenced by a slow degradation of compounds 1 & 2 to 3 & 4, respectively.

**TABLE 1.** HPLC[a] and UV Data for compounds **1** & **2** and their associated amides (**3** & **4**, respectively).

| Compound # | Retention Time / min. | $\lambda_{max}$ / nm |
|---|---|---|
| **1** | 29.0 | 239.8±0.3[b] |
| **2** | 33.7 | 237.9±0.2 |
| **3** | 10.6 | 243.4±0.1 |
| **4** | 10.5 | 240.7±0.3 |

a. Supelco[5] LC-18 ($C_{18}$) reverse phase column, 50% MeOH:$H_2O$ mobile phase.

b. ± standard deviation of the mean ($s_{\bar{x}}$; 5 replications); spectra were collected on the HPLC with a diode array detector.



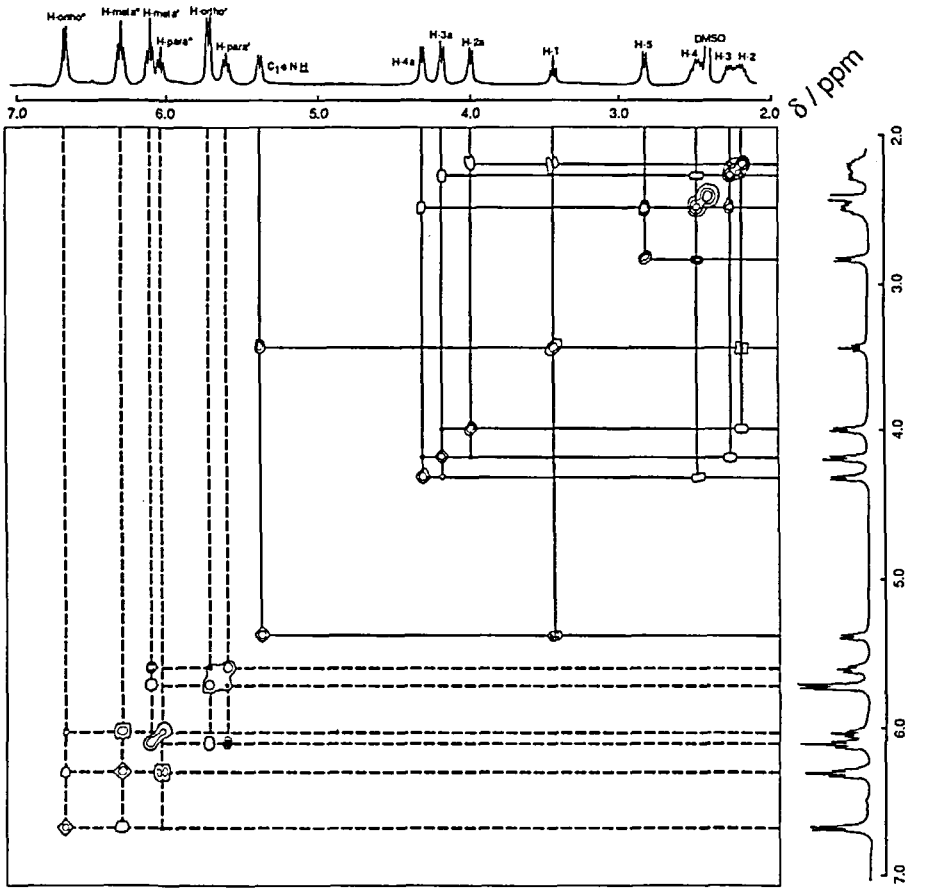



**FIG. 3.** 400 MHz proton shift-correlated 2D NMR (homonuclear COSY)[7] experiment of a saturated solution of compound **1** in DMSO-$d_6$ at 295 K.

the sugar's $C_5$ proton (Table 2; H-5). Based upon the position of off-diagonal resonances we assigned all the remaining pyranose ring protons including the OHs. The assignment of the proton NMR spectrum clearly demonstrated that compound 1 was not a 1-*amino*-1-deoxyketose as might be expected[4] to form (Fig. 1) by an Amadori rearrangement.

Having partially assigned the proton NMR spectrum of 1 we performed a heteronuclear COSY[10] experiment (Fig. 4) in order to assign the $^{13}C$ resonances. From the proton NMR assignments (Table 2) additional information on $^{1}H$-$^{13}C$ scalar connectivities (Fig. 4) enabled us to completely assign all but the aromatic region of the $^{13}C$ spectrum. In order to finish the assignment of the aromatic proton resonances for compound 1 we performed 2D nOe[11,12] NMR spectroscopy experiments (Table 3) with different mixing times. In these 2D nOe experiments the intensities of the off-diagonal resonances, which did not occur in the normal homonuclear COSY experiment, were considered measures of interproton distances.[12] Because of these dipolar interactions we assigned those aromatic proton resonances that were associated with the $C_1$-*N*-glycoside functionality. Table 3 indicated that there were strong dipolar interactions between H-1 ($\delta$ = 3.47 ppm) and H-ortho' aromatic ($\delta$ = 5.73 ppm) protons. Likewise there was a strong dipolar interaction between the H-ortho' and the amine N-H ($\delta$ = 5.37 ppm) protons. The 2D nOe data also indicated that there was a strong dipolar interaction between H-5 and H-1 which would only be feasible if 1 was in the $\beta$ anomeric configuration. Assuming that the off-diagonal peaks were real, these data completed the $^{1}H$ (Table 2) and $^{13}C$ (Table 4) spectral assignments.

It was of interest to note that (Table 3) strong off-diagonal nOe resonances existed between compound 1's —OHs and DMSO. We hypothesized that these off-diagonal peaks and their corresponding diagonal components represented a H-bonded *N*-phenyl(*N*-phenyl-β-D-glucopyranosylamine)uronamide:DMSO system. Evidence for this was as follows: [a] the

**TABLE 2.** Proton NMR Spectral Data for *N*-phenyl(*N*-phenyl-β-D-glucopyranosylamine)uronamide (1).

| | *Observed* | | | | *Calculated* | |
|---|---|---|---|---|---|---|
| | | $T_{1H}$ / s[b] | | | | |
| Proton | δ / ppm[a] | 270 MHz | 400 MHz | $J_{H-H}$ / Hz[c] | $J_{H-H}$ / Hz[d] | φ / deg[d] |
| H-1 | 3.47 | 0.25 | 0.61 | $J_{H_1-H_2}$=8.4 | 8.4 | 179° |
| H-2 | 2.26 | --- | --- | $J_{H_2-H_3}$=8.6 | 8.6 | 170° |
| H-2a | 3.98 | 0.84 | 1.46 | | | |
| H-3 | 2.35 | --- | --- | $J_{H_3-H_4}$=8.4 | 8.5 | 168° |
| H-3a | 4.28 | 0.84 | 1.42 | | | |
| H-4 | 2.57 | --- | --- | $J_{H_4-H_5}$=9.5 | 10.0 | 170° |
| H-4a | 4.31 | 0.85 | 1.37 | | | |
| H-5 | 2.86 | 0.31 | 0.77 | | | |
| $C_6$'s N-H | 9.06 | --- | --- | | | |
| H-ortho" | 6.63 | 1.09 | 1.84 | | | |
| H-meta" | 6.26 | 1.11 | 2.15 | | | |
| H-para" | 6.01 | 1.59 | 2.50 | | | |
| $C_1$'s N-H | 5.37 | 0.33 | 0.56 | | | |
| H-ortho' | 5.73 | 0.66 | 1.20 | | | |
| H-meta' | 6.08 | 1.02 | 1.86 | | | |
| H-para' | 5.61 | 1.05 | 2.08 | | | |
| DMSO | 2.49 | 1.67 | 3.27 | | | |

a. Chemical shifts are relative to $(CH_3)_4Si$ (0 ppm) at 20-23 °C.
b. $T_{1H}$s measured at 40 °C. Asymptotic standard errors were less than 5%.
c. Coupling constants (20-23 °C) were obtained by specific proton decoupling of near neighbors.
d. Calculations were performed using MacroModel™ version 1.1. The Karplus relation[13] used to calculate the dihedral angles, φ, were obtained from the energy-minimized structure.

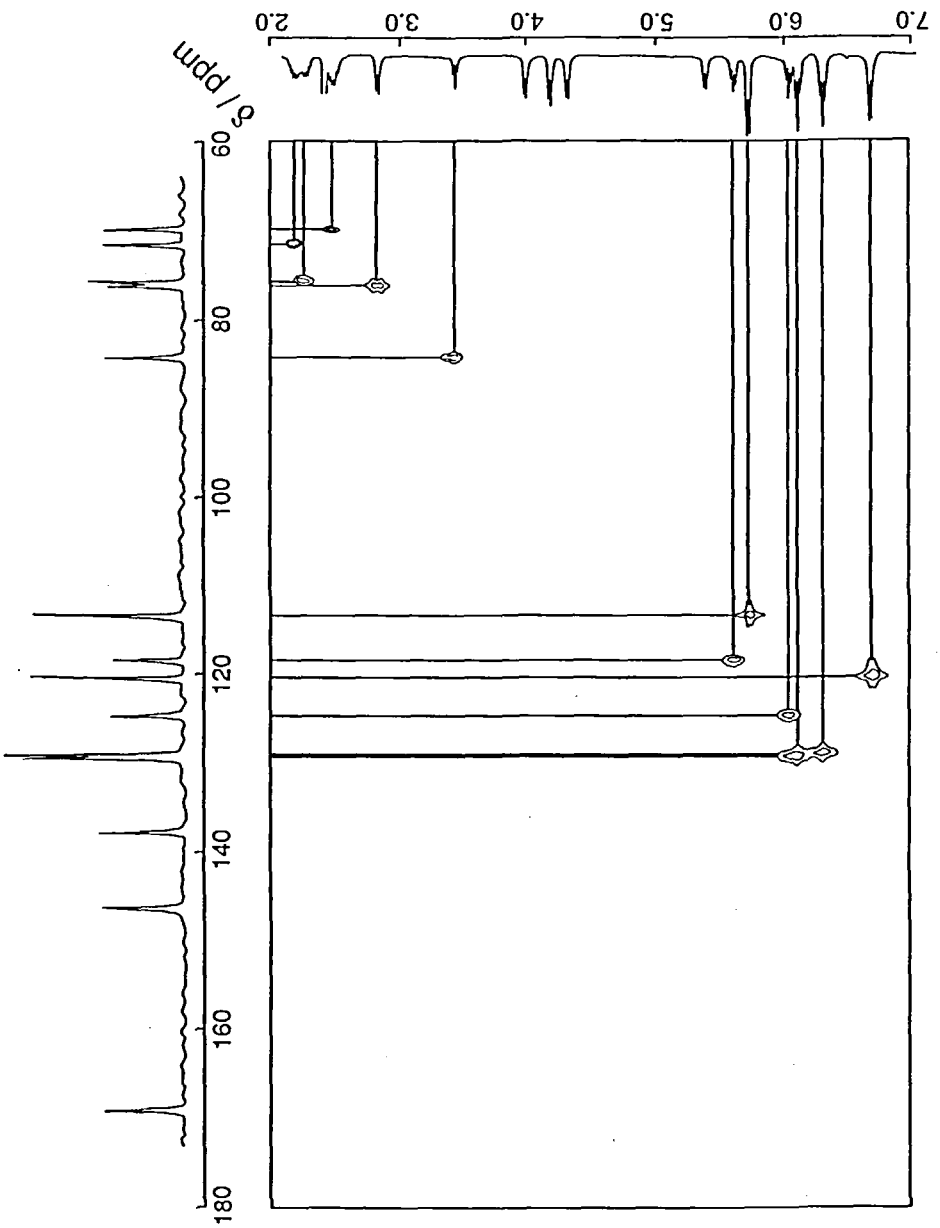
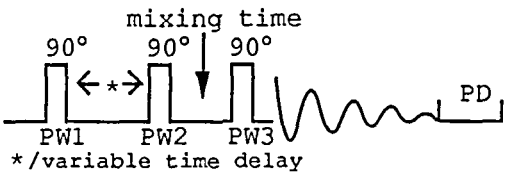



FIG. 4. 100 MHz $^{13}C-^{1}H$ shift-correlated 2D NMR (heteronuclear COSY)[10] experiment of a saturated solution of compound **1** in DMSO-$d_6$ at 295K.

**TABLE 3.** Apparent proton nOes[11] for *N*-phenyl(*N*-phenyl-β-D-glucopyranosylamine)uronamide (compound **1**) in DMSO.

| Proton assignment | *$^1$Hs with significant Dipolar Interactions* | | |
|---|---|---|---|
| | 100 | 300 | 750[a] :mixing time/ms |
| H-1 | --- | H-5 | H-5 |
| | --- | --- | H-3 |
| H-2a | H-1 | DMSO[b] | DMSO |
| H-3 | --- | H-5 | --- |
| H-3a | H-4 | H-4 | DMSO |
| | --- | DMSO[b] | --- |
| H-4a | H-3 | H-2a | H-2a |
| | H-5 | H-5 | DMSO |
| | --- | DMSO[b] | --- |
| $C_1$'s N-H | --- | H-2 | --- |
| H-ortho" | H-Para' (*w*)[c] | DMSO (*w*) | --- |
| | H-3a | --- | --- |
| | H-4 (*w*) | --- | --- |
| | $C_6$'s N-H | | |
| H-meta" | --- | DMSO (*w*) | H-Para' (*w*) |
| H-ortho' | --- | H-1 | $C_1$'s N-H |
| | --- | --- | H-1 |
| | --- | DMSO (*w*) | --- |

a. Pulse sequence:




| *phase* | | |
|---|---|---|
| PW1 | PW2 | PW3 |
| X | X | X |
| -X | Y | Y |
| X | -X | -X |
| -X | -Y | -Y |

b. The DMSO/-OH nOe persists without matrix symmetrization and in ROESY experiments

c. *w* = weak off-diagonal resonance

off-diagonal -OH resonances persisted in ROESY experiments; [b.] the relative intensities of the off-diagonal -OH resonances increased as a function of the fixed delay (Fig. 5) times. Of course, the solution structures of simple carbohydrates are sensitive to not only the size and location of substituents but also the polarity of the solvent.[14]

Due to potential problems with spurious off-diagonal resonances in 2D nOe experiments, we confirmed our aromatic $^1$H and $^{13}$C assignments with a natural abundance 2D double quantum

**TABLE 4.** $^{13}C$ NMR Chemical Shift Data for *N*-phenyl(*N*-phenyl-β-D-glucopyranosylamine)uronamide (compound **1**).

| Carbon assignment | δ / ppm[a] |
|---|---|
| C-1 | 86.1 |
| C-2 | 73.5 |
| C-3 | 77.9 |
| C-4 | 72.3 |
| C-5 | 78.2 |
| C-6 | 168.9 |
| C-1" | 138.7 |
| C-ortho" | 121.3 |
| C-meta" | 130.2 |
| C-para" | 125.9 |
| C-1' | 147.2 |
| C-ortho' | 114.8 |
| C-meta' | 130.5 |
| C-para' | 119.7 |

a. Chemical shifts are relative to $(CH_3)_4Si$ (0 ppm).



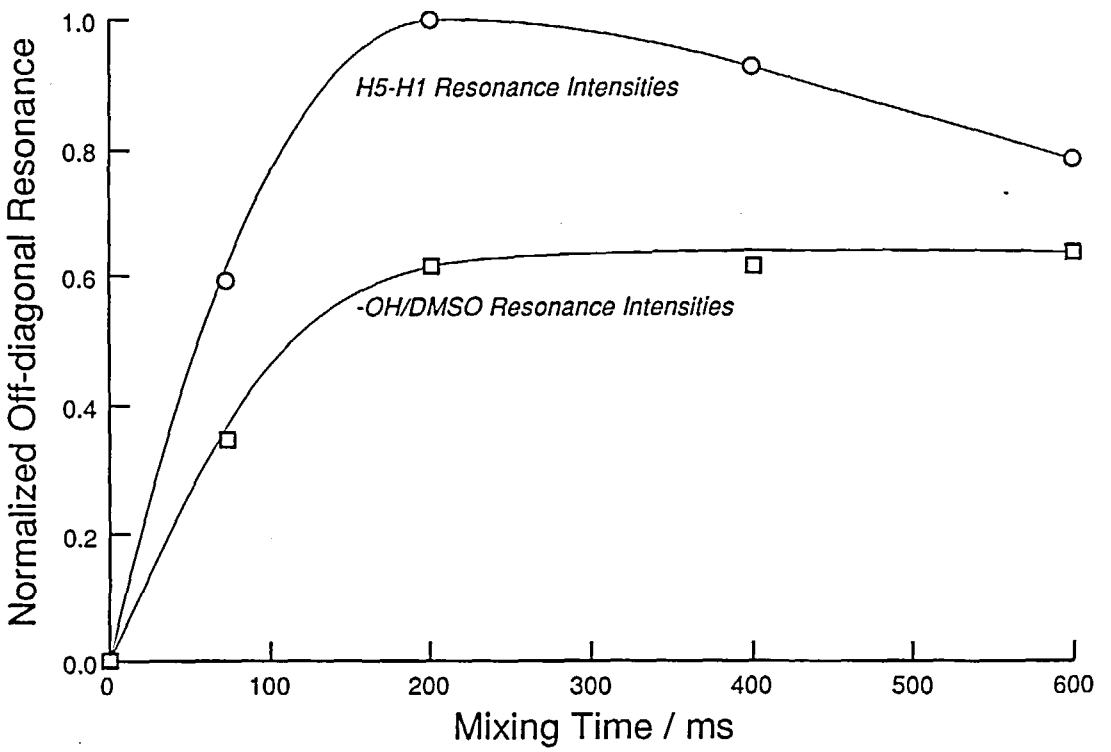



**FIG. 5.** Change in -OH/DMSO (average of 3 off-diagonal doublets; squares) and H-1/H-5 integrated intensity (average of 3 independent measures of the off-diagonal H-1 triplet; circles) at 400 MHz as a function of the fixed delay time in a ROESY experiment of compound **1** in DMSO-$d_6$ at 311K.

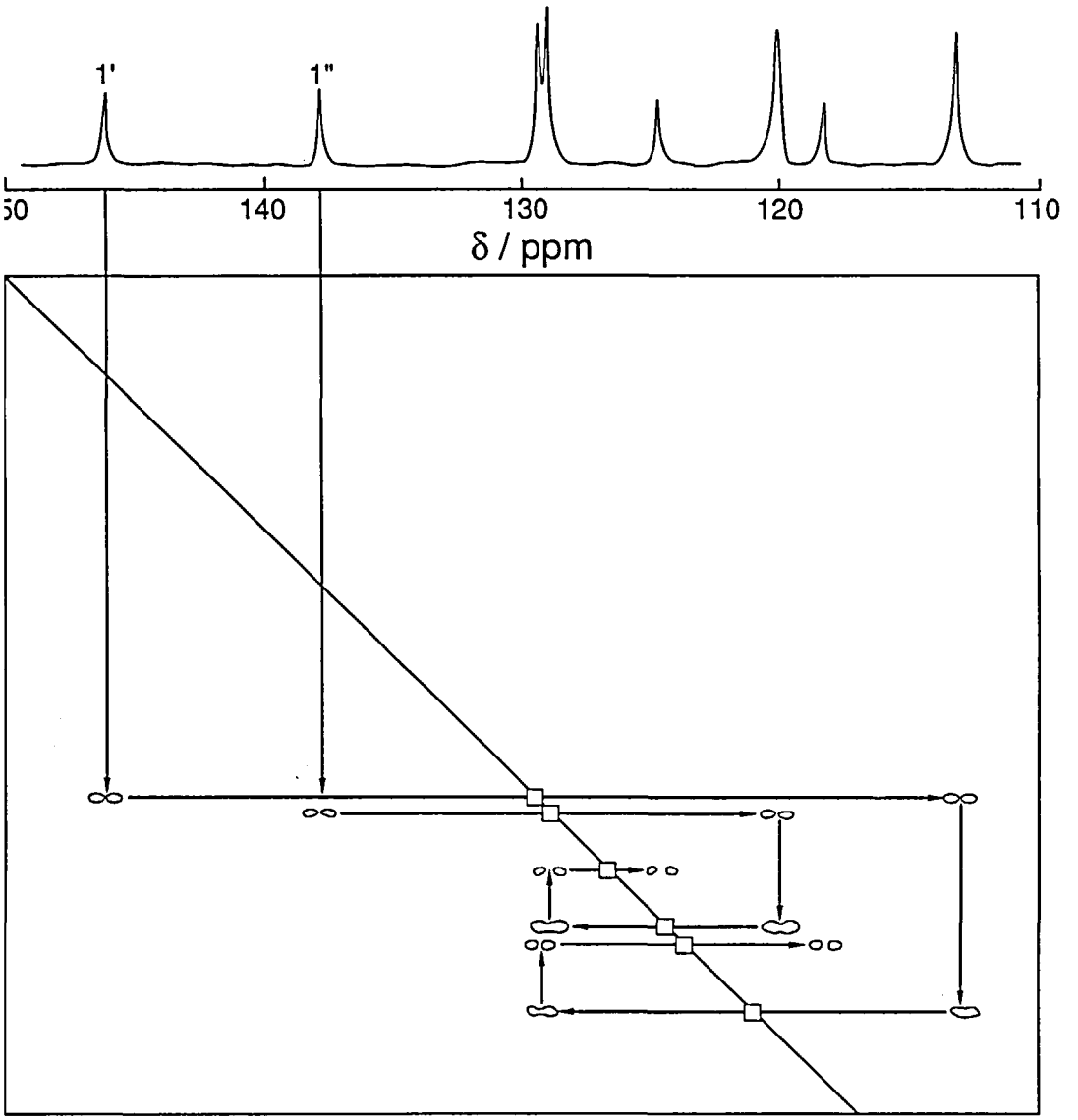





**FIG. 6.** 100 MHz $^{13}C$ 2D double quantum transfer experiment (2D INADEQUATE)[15,16] of the aromatic region of a saturated solution of compound **1** in DMSO-$d_6$ at 295 K. Resonance 1' and 1" were assigned based upon literature values[19] of various aromatic amines and amides.

transfer experiment (2D INADEQUATE[15,16]), as shown in Fig. 6. Artificially large off-diagonal 2D nOe components evolved[12] when there were weak dipolar interactions between spin populations with different correlation times ($\tau_c$; cf. ortho' & H-1 $T_1$s, Table 2). In the 2D INADEQUATE experiment,[17] the second dimension provided the double quantum transition[18] frequencies of each $^{13}C$-$^{13}C$ doublet. In Fig. 6 we have assigned the amide aromatic (1") and amine aromatic (1') quaternary resonances based upon literature[19] values. Thus, the 2D INADEQUATE experiment completely supported the previous $^1H$ and $^{13}C$ chemical shift assignments based upon COSY and 2D nOe techniques.

In order to confirm the conformation and configuration proposed for **1** it was necessary to deduce the scalar $^1H$-$^1H$ coupling constants (Table 2) by selective proton irradiation experiments. The fact that $J_{H1-H2}$ = 8.4 Hz supported the hypothesis that compound **1** was in the β anomeric configuration ($\varphi_{H1-H2} \cong 179°$). All the scalar coupling constants were ca. 9 Hz (i.e., $^1H$-$^1H$ dihedral angles ≅ 170-180°, Table 2); this argued that the pyranose ring's directly-bonded protons were axial and, thus, **1** existed in DMSO predominantly as the $^4C_1$ conformer.[20] This observation has also been tested and confirmed with energy minimization calculations ($\Delta H$ for $^4C_1$ = 5.43 kcal·mole$^{-1}$). We suspected that some of the weak apparent nOe interactions shown in Table 3 (i.e., Ortho"–Para' and Meta"–Para') were due to possible intermolecular interactions.[21] Other evidence for this was also noted in Table 2 where it was apparent that this compound behaved like a much larger molecule since the $T_{1H}$s at 400 MHz were significantly larger than those at 270 MHz. Work is in progress to understand these proposed intermolecular interactions further.

## EXPERIMENTAL

**Sample Preparation.** D-gluco- or D-galactopyranuronic acid (1.5 g) was dissolved in 25 mL of $H_2O$. Aniline (2 mL)

was added and the pH adjusted to 4.75 on a Radiometer[5] pH stat/titrator. Upon adding ca. 3 g of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) the pH stat was started and 0.1N HCl added automatically to the reaction mixture to maintain the pH at 4.75.[1] When no more titrant was needed to maintain a static pH the reaction was considered to be complete; the insoluble white precipitate was thoroughly washed with $H_2O$ which was subsequently removed by lyophilization. The **D**-glucuronic acid derivative was dissolved in hot EtOH and 2-4 mm needle-like crystals (decomposition point = 128-130 °C) formed overnight at room temperature. Anal. Calcd for $C_{18}H_{20}O_5N_2 \cdot H_2O$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.67; H, 6.53; N, 7.39. Several attempts were made to crystallize the **D**-galacturonic acid derivative without success; any work with this compound reported herein was performed with the amorphous form.

Preparation of the NMR samples (saturated solutions[21] were used in all the non-irradiation NMR experiments) was performed in a dry box with DMSO-$d_6$ which had been previously stored several days with molecular sieve pellets under dry $N_2$. Two DMSO-$d_6$-washed molecular sieve pellets were kept in the 5 mm NMR tubes to maintain the sample in a dehydrated state; the NMR tubes were closed and wrapped with parafilm to assist in the exclusion of $H_2O$ vapor.

**Kinetic Experiments.** Approximately 3 mg of each compound were dissolved in 10 mL of either 50% [v/v] MeOH:$H_2O$ or abs MeOH (not redistilled) and maintained at 40 °C in a New Brunswick Scientific PsycroTherm (100 rpm agitation) throughout the experiment. At times 0, 3, 10, 23 and 27 h, 100 µL of each solution were injected (5 µL injection loop) into a HP 1090 HPLC system (HP-85B computer system) with a Supelco LC-18 ($C_{18}$) reverse phase (15 cm; 5 µm particle size) column. 50% [v/v] MeOH:$H_2O$ was used as the mobile phase with a flow rate of 0.2 mL·min$^{-1}$. The various peaks were checked against standards of aniline, *N*-phenyl-**D**-gluco- or galacto-pyranuronamide.

**NMR Spectroscopy.** All 2D spectra shown were collected either on a JEOL GX-400 NMR spectrometer system operated at

ca. 100 MHz for $^{13}C$ and ca. 400 MHz for $^{1}H$ using 5mm probes (ERRC, Philadelphia, PA). Unless otherwise specified, all data were processed without zero-filling due to memory limitations. Computer line broadening was selected to be approximately equal to the resolution.

The homonuclear COSY proton spectrum was acquired using a (square) matrix of 256 x 1024 ($t_1$ x $t_2$) complex data points which represented a spectral width of 2 kHz for either dimension. For each $t_1$ spectrum collected, 8 scans were acquired. A sine-bell apodization function (window function) was used to process these data. The proton 90° pulse width was 6 μs. 2D nOe experiments were performed identically to the homonuclear COSY for protons but with a mixing time which was added prior to collection of the FID (see Table 3) during $t_2$.



The heteronuclear COSY experiment was performed with a complex data matrix of 256 x 1024 ($t_1$ x $t_2$) data points representing spectral widths of 2 kHz ($\nu_1$) and 8503.4 Hz ($\nu_2$) for $^{1}H$ and $^{13}C$ frequencies, respectively. A Gaussian window function was used to process these data. For each $t_1$ spectrum collected 32 transients were acquired. The 90° pulse widths were 9 μs and 36 μs for $^{13}C$ and $^{1}H$, respectively. The fixed delays, $\{2J_{CH}\}^{-1}$ and $\{4J_{CH}\}^{-1}$, were 3.33 and 1.67 ms (e.g., $J_{CH}$ = 150 Hz).

The 2D INADEQUATE experiment was performed with a data matrix of 512 x 512 ($t_1$ x $t_2$) complex data points representing spectral widths of 7407.4 Hz ($\nu_1$) and 3703.7 Hz ($\nu_2$). An exponential window function was used to process these data. For each $t_1$ spectrum collected 640 transients were acquired. The 90° pulse width was 9.5 μs. The fixed delay, $\{4J_{CC}\}^{-1}$, was 4.39 ms (e.g., $J_{CC}$ = 57 Hz).

Proton $T_1$ inversion recovery experiments were performed also on JEOL NMR spectrometers (JEOL, Peabody, MA) operated at 400 and 270 MHz at 40 °C. The proton full-power 90° pulse was 6.3 μs at 400 and 4 μs at 270 MHz in a 5 mm probe. Each $\tau$ value was signal averaged for 64 acquisitions with 16 dummy scans. Peak intensity data were fitted to an exponential

function, $M = M_0\{1-2\exp[-(\tau - \tau_0)/T_{1H}]\}$, to obtain the $T_1$s; in this expression $M = -M_\infty$ at $\tau_0$. All asymptotic standard errors for this calculation were $T_{1H} \pm \{ \leq 5\% \}$.

ROESY data (ca. 40 mM **1**) were collected using a JEOL GSX-400 NMR spectrometer (JEOL, Peabody, MA) at 40° C. The proton full-power 90° pulse was 10.5 μs. Acquisition data sets consisted of 2048 complex points for $t_2$ and 64 acquisitions for each $t_1$ data set. A spin-lock field of 3 kHz, 1 kHz off-resonance from the average chemical shifts of the residual DMSO protons and the —OHs, was used for mix times of 75, 200, 400 and 600 ms. The data sets were zero-filled to 4096 $t_2$ points and 2048 points for $t_1$. A phase-shifted sine-bell was used as the apodization function.

**Energy Minimization Calculations.** All calculations were performed using MM2 force field within MacroModel™ version 1.1. Initial structures were obtained from the MacroModel carbohydrate library and modified appropriately. Coupling constants (Table 2) were calculated from the dihedral angles using the Karplus relations.[13]



## ACKNOWLEDGEMENTS

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21. Ad Bax personal communication. A high concentration of **1** could account for some of the unusual (Table 3: H-ortho"-H-para' & H-meta"-H-para') phenyl ring proton nOe interactions due to intermolecular complexation of the sugars. Upon repeating the 2D nOe experiments with a much more dilute (42 mM) sample, the off-diagonal inter-ring H-ortho"-H-para', as well as the DMSO-OH resonances, were still apparent.