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Conformational Studies by NMR of the Antimicrobial Peptide, Drosocin, and Its Non-Glycosylated Derivative: Effects of Glycosylation on Solution Conformation[†]

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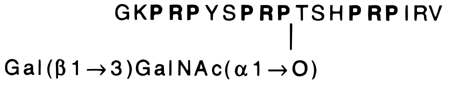
ABSTRACT: Drosocin is a cationic 19 amino acid peptide secreted by *Drosophila* in response to septic injury. The sequence (GKPRPYSPRPTSHPRPIRV) contains six Pro and four Arg residues which are incorporated into three repeated triplet sequences Pro-Arg-Pro. The peptide is glycosylated at Thr11 and has potent antimicrobial activity. This activity is markedly reduced on deglycosylation, but a structural basis for this has not been previously established. In the current study, the solution conformations of drosocin and its non-glycosylated derivative were determined by NMR spectroscopy and structure calculations. The NMR and structure studies showed that the peptides have significant populations of essentially random coil conformations in aqueous solution. Addition of 50% trifluoroethanol causes the development of small populations of folded conformations, mainly in the form of turns. In particular, turn elements occur near residues 4−7, 10−13, 17, and 18. No substantial difference was detected in the predominantly random coil conformation of the glycosylated and non-glycosylated forms, but there are subtle differences in the small populations of folded conformers. In particular, the turn at residues 10−13 tends toward a more extended structure on glycosylation, while there is some tightening of the downstream turn at residues 17 and 18. There are a significant number of nuclear Overhauser enhancement contacts between the sugar moiety and the peptide near the glycosylation site, consistent with a close association between them. Despite this close association, the pK_a of H13, which is proximate to the glycosylation site, was found to be unaffected by glycosylation.

Insects display a remarkable resistance to infection by microorganisms. In part, this is the result of the rapid synthesis of antimicrobial peptides following bacterial infection or septic injury (*1*). At present there are four known classes of insect antimicrobial peptides: the cecropins, insect defensins, a glycine-rich family, and a proline-rich family (*2*). Drosocin, a peptide originally isolated from *Drosophila*, belongs to the last of these families which also includes pyrrhorocorin (*Pyrrhocoris apterus*), apidaecin Ia, Ib, and II and abaecin (*Apis mellifera*), formaecin 1 and 2 (*Myrmecia gulosa*), lebocin I, II, and III (*Bombyx mori*), and diptericin (*Phormia terranovae*) (*3*−*5*). Drosocin has antibacterial activity primarily directed against gram-negative strains. The peptide consists of 19 amino acid residues, nearly one-third of which are prolines, as shown in the following sequence.



$$\begin{array}{l} \text{GK}\mathbf{PRP}\text{YS}\mathbf{PRP}\text{TSH}\mathbf{PRP}\text{IRV} \\ \qquad\qquad\qquad\quad | \\ \text{Gal}(\beta 1\rightarrow 3)\text{GalNAc}(\alpha 1\rightarrow \text{O}) \end{array}$$

In the major chromatographic fragment of the naturally occurring peptide, T11 is *O*-glycosylated by a disaccharide comprised of *N*-acetylgalactosamine and galactose moieties (*6*). The terminal Gal is 3-linked to GalNAc, which is directly linked through its anomeric carbon to the hydroxyl group of T11. Previous studies (*3, 5, 7*) have shown that glycosylation of drosocin is very important for biological activity, with the non-glycosylated peptide having an activity up to eight times less than the glycopeptide when tested against sensitive bacterial strains. Drosocin bearing only a monosaccharide is more active than the non-glycosylated form but less active than the disaccharide derivative. Analysis of truncated isoforms of drosocin has shown the first five and the last two residues to be essential for biological activity. A peptide bearing all D-amino acids had an activity 50−150 times less than the native isomer. The latter result coupled with the fact drosocin takes 24 h to kill bacteria suggests a reaction mechanism involving interaction with a stereospecific receptor. Similar results have also been observed for a number of other members of this family (*5*). By contrast, studies of members of the cecropin and defensin families have concluded that these peptides act through a lytic/ionophoric, non-stereoselective mechanism which kills the cell within minutes (*8*, *9*).

In addition to its high proline content, drosocin is highly positively charged owing to the incorporation of four arginines, a histidine, a lysine, and no negatively charged residues. It is interesting that all of the prolines and all but one of the arginine residues are arranged in triplet repeats, PRP, which occur three times in the sequence (shown in bold in the sequence above). Several other members of the proline-rich family also have the PRP triplet within their primary

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sequences. At present, the significance of this is unknown, but it may have an important bearing on the three-dimensional structures of the peptides at their site of action.

The mechanism by which the sugar residues affect biological activity is also not yet understood; however, several possibilities exist. For example, the sugars might be directly involved in receptor binding interactions, or they could alter the conformation of the peptide backbone, thereby affecting its receptor binding. Sugar residues may influence the dynamics of the peptide and hence influence binding via entropic effects (*10*, *11*). Alternatively, glycosylation could result in the modification of physicochemical properties of the peptide.

Conformational studies have so far been restricted to analysis of drosocin and its non-glycosylated derivative by circular dichroism (CD). CD studies in both aqueous and membrane-mimicking solvents concluded there was little difference in conformation between the two forms of the peptide (*3*). However, CD is mainly used to detect the overall secondary structure content; hence, subtle conformational changes occurring in a small percentage of the peptide population may not be detected. By contrast, NMR is a technique which can detect local conformational changes. The aim of the current NMR study was to determine and compare the structures of synthetic drosocin and its non-glycosylated derivative with a view to understanding the role of the sugar residues in biological activity. In this regard drosocin appears to be a good model for studying the conformation-modifying effect of short O-linked carbohydrates in naturally occurring glycopeptides and glycoproteins.

The current widespread interest in the potential therapeutic applications of both natural and synthetic glycopeptides means this study has much broader implications than for drosocin alone. The attractive features of glycopeptides when compared to their non-glycosylated parent analogues include increased solubility and oral availability (*12*, *13*), increased serum half-life (*14*), broader biological activity spectrum (*15*, *16*), and a more diverse set of conformational variants than is available from peptides alone (*17*, *18*).

Among recent studies on glycopeptides two general mechanisms explaining the role of sugar residues in determining conformation have emerged. One proposal is that the primary role of the sugars is to induce conformational changes by simply reducing the number of conformations available to the otherwise flexible peptide (*10*, *19*, *20*). In most cases studied so far, the peptide backbone demonstrates a tendency to bend away from the sugar residue. Therefore, the sugar can be thought of as turn-inducing, albeit in a rather nonspecific way. However, it has been demonstrated that the nature of the sugar can influence the nature of the turn (*10*, *21*). The second theory is that there are specific interactions, most notably involving H-bonding between sugar and peptide, that help form "glyco-turns" (*22*). The sugar−peptide interaction theory received recent support from NMR measurements of a glycosylated tripeptide where a specific sugar−peptide turn NOE[1] was measured (*23*, *24*). However, this latter study used a very short model peptide with solution and temperature conditions not very applicable to natural products.

The current study provides new input into this debate because we demonstrate glycosylation is not always turn-inducing but can, in some cases, result in local straightening of the peptide backbone.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Peptides were assembled on solid phase using a Milligen 9050 automated peptide synthesizer employing standard Fmoc methodology (*25*). The glycosylated threonine residue was incorporated as Fmoc-Thr-(β-D-Ac$_4$-Gal-(1→3)-α-D-Ac$_2$GalNAc-1)-OH (purchased from Bachem California, Torrance, CA) in the same manner as unmodified amino acids. Peptides were cleaved from the solid support with trifluoroacetic acid in the presence of scavengers and were purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The sugar-protecting acetyl groups were removed by a 2 min treatment with 0.1 M NaOH, and the glycopeptide was rechromatographed (*26*). The final products were characterized by amino acid analysis, capillary zone electrophoresis, and matrix-assisted laser desorption/ionization mass spectroscopy. The HPLC showed single peaks with the glycopeptide eluting 0.6 min before the non-glycosylated analogue, and the mass spectra revealed single ions at m/z 2198 (non-glycosylated drosocin) or m/z 2565 (glycosylated drosocin) (*3*).

^{1}H NMR Experiments. ^{1}H NMR spectra were recorded on Bruker DMX 750 MHz and DRX 500 MHz spectrometers at temperatures in the range of 280−313 K. Two-dimensional NMR spectra were recorded in the phase-sensitive mode using time proportional phase incrementation for quadrature detection in the f_1-dimension (*27*, *28*). The water proton signal was suppressed by low-power irradiation during the relaxation delay (1.8 s) and during the mixing time of NOESY experiments (in nongradient experiments). Solvent suppression for NOESY and TOCSY experiments was achieved using presaturation. Spectra were referenced to DSS.

TOCSY spectra, recorded using an MLEV-17 mixing scheme (*29*) with mixing times of 80 or 120 ms, were used in the assignment process to identify side chain spin systems. DQF−COSY experiments (*30*) were recorded in an attempt to measure $^3J_{\mathrm{NH-\alpha H}}$ coupling constants (*27*). 2D spectra were collected over 4096 data points in the f_2-dimension. In most cases 512 increments of 48 scans were acquired over a spectral width corresponding to 11 ppm for both dimensions. Up to 64 scans per increment were acquired for NOESY experiments, and 16 scans per increment and 512 slices were acquired for TOCSY experiments.

The data were processed on a Silicon Graphics (SGI 4D/30) computer using the UXNMR software package. The f_1-dimension was zero-filled to 4096 real data points, with f_1- and f_2-dimensions being multiplied by a squared sine function and Gaussian function, respectively, prior to Fourier transformation. Polynomial base-line correction was used in selected regions to improve the appearance of the spectrum.

^{1}H−^{13}C NMR Experiment. A ^{1}H−^{13}C HSQC spectrum (*31*−*33*) was recorded for the drosocin sample in 50% TFE/water at 290 K to assist with the assignment of some of the

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[1] Abbreviations: NOE, nuclear Overhauser enhancement; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TOCSY, total correlated spectroscopy; DQF-COSY, double quantum filtered correlated spectroscopy; NOESY, NOE spectroscopy; HSQC, heteronuclear single quantum filtered correlated spectroscopy; TFE, trifluoroethanol.

sugar protons. Spectral widths in the ^{1}H and ^{13}C dimensional were 8993 and 22638 Hz, respectively. A set of 2048 data points as acquired with 300 f_1 increments and 512 scans per increment.

Structure Calculations. The strengths of peaks were determined by counting contour levels in the 300 ms NOESY spectrum at 283 K. Peaks were classified as either strong, medium, weak, or very weak. These classifications corresponded to upper bounds of 2.7, 3.5, 5.0, and 6.0 Å, respectively (*34*). The upper limits of nonstereospecifically assigned methylene, methyl, and aromatic protons were adjusted using standard pseudoatom corrections (*35*).

Three-dimensional structures were calculated using simulated annealing and energy minimization protocols with XPLOR (version 3.1; *36*, *37*). An ab initio simulated annealing protocol was used to generate a set of 100 structures starting from template structures with randomized φ and ψ angles and extended side chains (*38*). This protocol consisted of a total of 30 ps of molecular dynamics (1000 K) divided into 20 ps with a low weighting on the NOE restraints and low repel force constants, followed by 10 ps with an increased force constant on the NOEs. Finally, the system was cooled to 100 K over 12 ps of molecular dynamics during which the repel force constant was gradually increased. The structures were then subjected to 2000 cycles of energy minimization by using the conjugate gradient Powell algorithm under the influence of the CHARMm force field (*34*, *39*). Parameter and topology files for the disaccharide were obtained by submitting a preliminary PDB file of arbitrary geometry to the program HIC−UP (Hetero-compound Information Centre, Uppsala University) (*40*). This program generated parameter and topology files on the basis of the standard XPLOR force field. Minor alterations were then made to the nomenclature within these files to incorporate them into XPLOR format.

RESULTS

Spectral Assignment. Although drosocin comprises only 19 amino acids, it presented a significant challenge to conformational analysis by NMR because of the triplet repeat sequence of PRP occurring three times within the sequence. ^{1}H NMR spectra of sub-millimolar concentrations of drosocin and its non-glycosylated derivative were recorded at 500 and 750 MHz at a range of temperatures and pH values. Preliminary spectra recorded in H_2O generally had poor dispersion and did not indicate the presence of any significant structure. Subsequent spectra were recorded in 50% TFE/water for comparison with previous CD data recorded in mixed TFE/water solution (*3*).

The TOCSY spectra were used to identify amino acid spin systems, and these were sequentially assigned by reference to NOESY spectra. In general, the spectra are relatively well-dispersed despite the presence of the multiple PRP triplet repeats. For example, in drosocin the amide chemical shifts of R4, R9, and R15 are mutually resolved by >0.15 ppm between any pair despite each arginine being the central residue of a PRP sequence. The range of shifts for these arginines is slightly smaller in the non-glycosylated peptide but still sufficiently resolved for unambiguous assignment. The range of amide chemical shifts of all residues in both peptides is approximately 1 ppm.

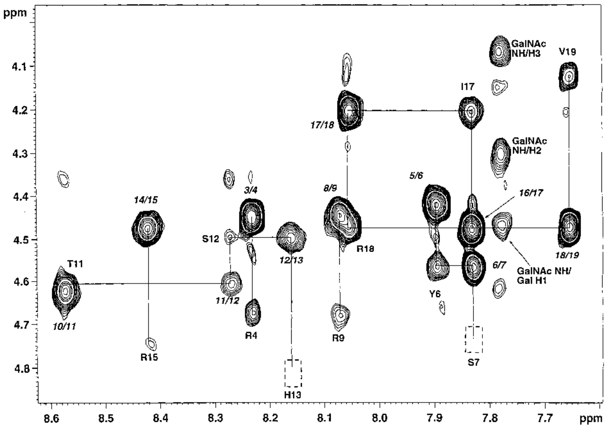

FIGURE 1: Fingerprint region of the NOESY spectrum of glycosylated drosocin (50% TFE/water, 283 K, pH ~7.0, 750 MHz) showing intraresidual and sequential NH−αH NOE connectivities as well assignments of the sugar residues.

The fingerprint region of the NOESY spectrum of drosocin, shown in Figure 1, illustrates the sequential connectivities used in establishing residue specific assignments. The sequential $d_{\alpha N}(i,i+1)$ NOE connection pathway is broken six times as a result of the six proline residues which lack NH protons. Despite this, unambiguous assignment of the entire sequence was possible by the identification of $d_{\alpha\delta}(i,i+1)$ NOEs between Pro and the preceding residue. $d_{\beta N}(i,i+1)$ connectivities were also observed for significant stretches of the sequence, and these helped to confirm the sequential assignments. In addition to aiding with assignments, the observation of strong $d_{\alpha\delta}(i,i+1)$ NOEs also confirmed that the peptide bonds preceding all of the proline residues were predominantly in the trans conformation.

The resonances of the sugar residues of drosocin were assigned primarily from TOCSY spectra. The amide proton of GalNAc yielded strong TOCSY cross-peaks to two signals at 4.31 and 4.07 ppm which were assigned to H2 and H3. Their shifts were very close to literature values for model oligosaccharides (*41*; mean values 4.31 and 4.04 ppm), and the TOCSY intensities were consistent with expected large couplings of NH to H2 and H2 to H3. The anomeric proton of GalNAc was well-resolved at 5.07 ppm and exhibited cross-peaks to the tentatively assigned H2 and H3 resonances in the high-field quadrant of the TOCSY spectrum. In a TOCSY spectrum recorded at 310 K there was a cross-peak to H1, as well as H2 and H3, at the NHAc proton frequency. This unambiguously confirmed these assignments. The remaining GalNAc protons were assigned by analogy with model compounds and with the aid of a ^{13}C−^{1}H HSQC spectrum (Figure 2).

The terminal Gal was difficult to assign from homonuclear 2D spectra as many of the sugar signals were overlapped with peptide signals, particularly from the proline residues. Again, however, by analogy with model compounds and the HSQC spectrum, it was possible to assign H2−H5 of the Gal residue (*42*). H1 was subsequently identified from the aliphatic region of the NOESY spectrum. The ^{1}H chemical shifts of both peptides are summarized in Table 1. Table 2 gives the ^{1}H and ^{13}C assignments for the sugar residues in drosocin.

The chemical shifts of the peptide backbone protons of drosocin and its non-glycosylated derivative were compared

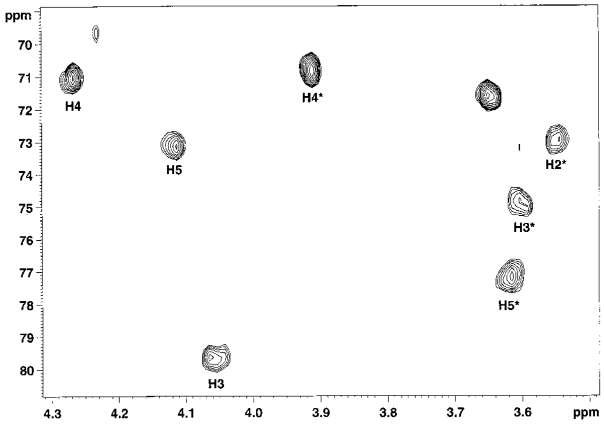

FIGURE 2: Region of the ^{13}C$-^{1}$H HSQC spectrum of drosocin showing assignment of selected sugar resonances.

Table 1: ^{1}H NMR Chemical Shifts of Drosocin and Its Non-Glycosylated Analogue in 50% TFE at 283 K (Relative to DSS, ppm)

residue	NH	αH	βH	other
GLY1		(3.86)		
LYS2		4.65	1.89	γCH$_2$, 1.53; δCH$_2$, 1.75; ϵCH$_2$, 3.04
	(8.56)	(4.66)	(1.91)	(γCH$_2$, 1.56; δCH$_2$, 1.77)
PRO3		4.45	2.07, 2.32	γCH$_2$, 1.91, 2.07; δCH$_2$, 3.66, 3.87
		(4.47)	(2.08, 2.33)	(γCH$_2$, 1.91, 2.08; δCH$_2$, 3.67, 3.88)
ARG4	8.23	4.68	1.71, 1.81	γCH$_2$, 1.71; δCH$_2$, ~3.24
	(8.24)	(4.68)	(1.73, 1.82)	(γCH$_2$, 1.73; δCH$_2$, 3.25)
PRO5		4.42	2.02, 2.26	γCH$_2$, 1.91, 2.02; δCH$_2$, 3.62, 3.76
		(4.43)	(2.03, 2.27)	(γCH$_2$, 1.94, 2.03; δCH$_2$, 3.63, 3.77)
TYR6	7.90	4.57	2.96, 3.07	3,5H, 6.83; 2,6H, 7.12
	(7.90)	(4.58)	(2.97, 3.07)	(3,5H, 6.84; 2,6H, 7.12)
SER7	7.83	4.74	3.79	
	(7.82)	(4.75)	(3.80)	
PRO8		4.45	2.03, 2.31	γCH$_2$, 1.98, 2.03; δCH$_2$, 3.68, 3.81
		(~4.43)	(2.04, 2.32)	(γCH$_2$, 1.98, 2.04; δCH$_2$, 3.63, 3.72)
ARG9	8.07	4.68	1.88	γCH$_2$, 1.72; δCH$_2$, ~3.24
	(3.14)	(4.70)	(1.90)	
PRO10		4.63	2.10, 2.36	γCH$_2$, 2.00; δCH$_2$, 3.65, 3.82
		(4.52)	(2.08, 2.35)	(γCH$_2$, 1.91, 2.08; δCH$_2$, 3.68, 3.82)
THR11	8.57	4.61	4.36	γCH$_3$, 1.36
	(8.06)	(4.42)	(4.32)	(γCH$_3$, 1.22)
SER12	8.27	4.50	3.82, 3.86	
	(8.15)	(4.50)	(3.88)	
HIS13	8.16	4.83	3.05, 3.17	2H, 7.68; 4H, 7.14
	(8.24)	(4.94)	(3.13, 3.23)	(4H, 7.25)
PRO14		4.47	2.03, 2.33	γCH$_2$, 2.03; δCH$_2$, 3.42, 3.79
		(4.48)	(2.03, 2.33)	(γCH$_2$, 2.03; δCH$_2$, 3.48, 3.79)
ARG15	8.42	4.75	1.79, 1.93	γCH$_2$, 1.75; δCH$_2$, ~3.25
	(8.31)	(4.77)	(1.78, 1.94)	(γCH$_2$, 1.78; δCH$_2$, 3.25)
PRO16		4.50	2.05, 2.28	γCH$_2$, 1.96, 2.05; δCH$_2$, 3.61, 3.80
		(4.50)	(2.06, 2.30)	γCH$_2$, 1.98, 2.06; δCH$_2$, 3.66, 3.81
ILE17	7.83	4.21	1.85	γCH$_2$, 1.18, 1.53; γ-δCH$_3$, 0.94
	(7.85)	(4.22)	(1.86)	(γCH$_2$, 1.20, 1.55; γ-δCH$_3$, 0.95)
ARG18	8.06	4.47	1.77, 1.90	γCH$_2$, 1.62; δCH$_2$, 3.22
	(8.05)	(4.48)	(1.78, 1.92)	(γCH$_2$, 1.64; δCH$_2$, 3.22)
VAL19	7.66	4.13	2.13	γCH$_3$, 0.91
	(7.67)	(4.14)	(2.14)	(γCH$_3$, 0.93)

to each other as well as to random coil chemical shifts (*43*). It is well-established that differences of αH chemical shifts from random coil values provide a first indication of the presence of secondary structure (*44*, *45*). These so-called secondary shifts are summarized in Figure 3a, and it is clear that for most residues in either peptide there is little deviation (<0.1 ppm) from random coil αH chemical shifts. This suggests that the peptides are predominantly not structured. Large secondary shifts occur for residues K2, R4, S7, R9, H13, and R15, but these all precede a proline and the deviation from random coil may be attributed to the well-known local sequence effect of prolines (Pro causes on average a 0.29 ppm downfield shifts of adjacent residues).

Table 2: ^{1}H and ^{13}C Shifts for the Sugar Residues in Drosocin in 50% TFE at 290 K (Relative to DSS, ppm)

proton chem shifts	GalNAc		Gal	
	^{1}H	^{13}C	^{1}H	^{13}C
H1	5.00		4.47	
H2	4.27	50.7	3.55	73.0
H3	4.05	79.6	3.60	74.9
H4	4.26	71.1	3.91	70.9
H5	4.11	73.2	3.62	77.2
H6				
NH	7.78			
NAc	2.01			

In general, the αH chemical shifts (Figure 3B) for the two peptides are identical to within 0.02 ppm except for P10, T11, and H13, suggesting there is no major conformational change induced by glycosylation. Amide proton chemical shifts are less useful as markers of conformational changes than αH shifts, but they do provide some insight into substitution effects and are particularly sensitive to hydrogen bonding interactions. Figure 3B shows that apart from the expected local effects (i.e., at T11, S12, H13) there is little change in the amide shifts of nonproximate residues with the exception of R15. The slight change exhibited by this residue may be the result of a hydrogen bonding interaction that occurs in the glycosylated peptide but not in the non-glycosylated peptide.

There are few changes in side chain proton chemical shifts on glycosylation, except for residues near T11. The most marked effect is on the side chain of T11. The methyl group resonance of T11 is moved downfield by 0.14 ppm upon glycosylation. In addition, the β-carbon has a ^{13}C-shift of 78.4 ppm which is downfield from the expected shift of between 67.9 and 68.3 ppm (*46*).

While chemical shifts are a useful indicator of secondary structure, it is difficult to detect the possible presence of small populations of structured forms among an ensemble of largely unstructured species. Therefore, other structural parameters were also examined. The NOE patterns of the two peptides were measured and are reported below.

NOE Connectivities. Sequential NOE connectivities observed for both peptides are summarized in Figure 4. Strong $d_{\alpha N}(i,i+1)$ NOEs, which are generally observed in extended structures (*47*, *48*), occur along most of the sequence. $d_{NN}(i,i+1)$ NOE connectivities, which are indicative of helical or turn elements of structure, were observed for only a few residues in drosocin, including Y6/S7, I17/R18, and R18/V19. These NOEs are also present in the non-glycosylated form and suggest there is local structure at these sites in both peptides. However, these sites of local structure most likely occur in a subpopulation of the predominantly disordered solution ensemble of conformers. The presence of local structure near these residues is supported by $d_{\alpha N}(i,i+2)$ NOEs at residues 5−7 and 17−19, although at the latter site the $d_{\alpha N}(i,i+2)$ NOE is quite weak in drosocin and absent in the non-glycosylated form. The turn propensity is clearly more pronounced near residues 5−7 than 17−19.

In contrast to these similarities between the two peptides there is a major difference in that there are additional $d_{NN}(i,i+1)$ NOEs in the stretch T11 to H13 in the non-

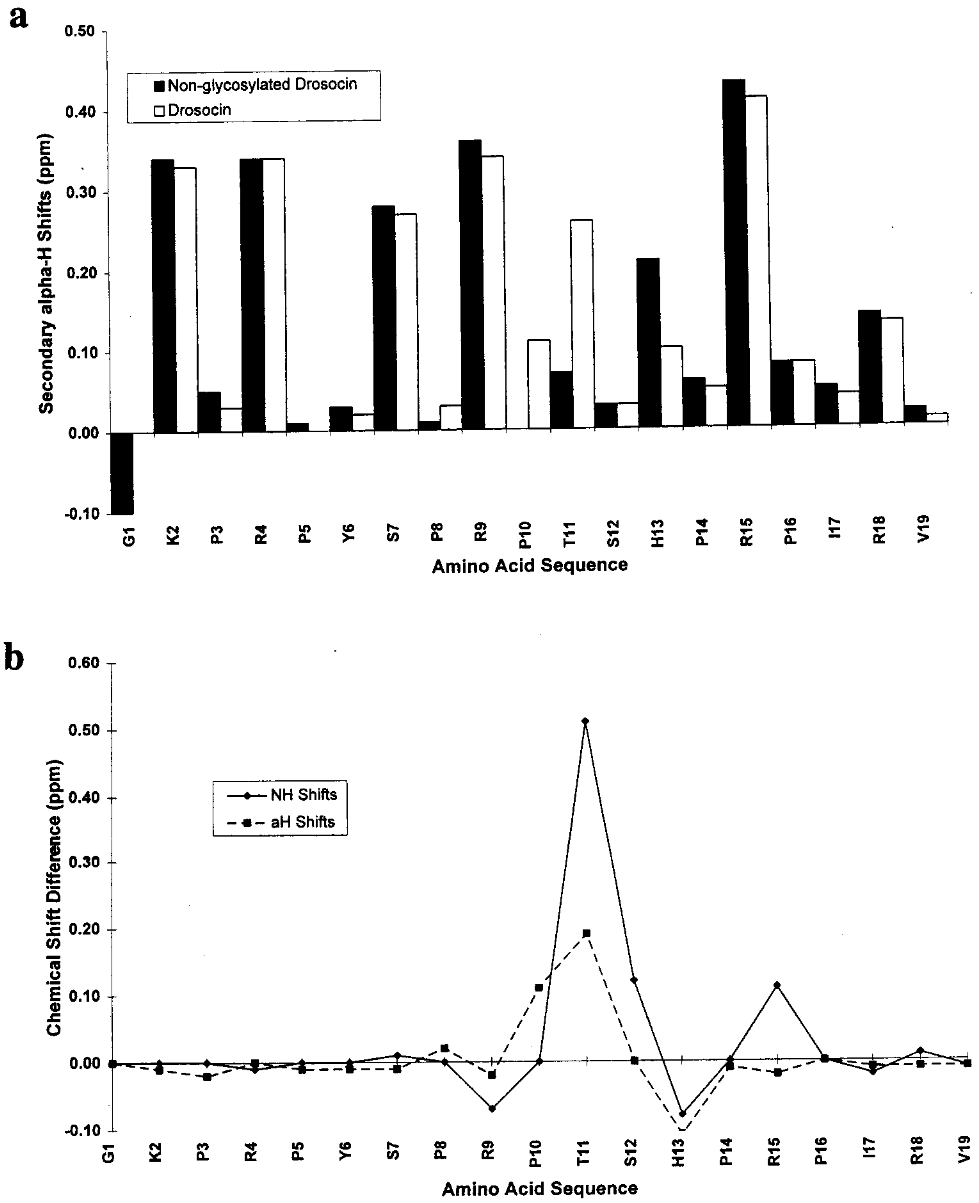

FIGURE 3: (a) Deviation of drosocin αH chemical shifts from their "random coil" (*43*) values in 50% TFE at 283 K. (b) Chemical shift differences between the glycosylated and non-glycosylated forms of drosocin.

glycosylated peptide. The latter are directly adjacent to the glycosylation site and provide an indication of a local conformational change on glycosylation. A $d_{\alpha N}(i,i+2)$ connectivity is also observed in the non-glycosylated form at residues 11−13 but not in drosocin itself. Thus, the data indicate there is at least a small population of conformers with a turn near T11 in the non-glycosylated derivative, but the propensity for a tight turn is reduced on glycosylation. This is a very significant finding as most previous studies have found that sugars tend to occur in, and cause enhancement of, turn regions of peptides and proteins (*19, 49*). The loss of $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+2)$ NOEs suggests the backbone is in a more extended conformation in glycosylated drosocin. However, some backbone-sugar NOEs (see below) suggest a degree of folded structure is maintained, although it is quite different from in the non-glycosylated form.

It is interesting to note the two to three residues immediately following each PRP repeat are remarkably similar as, unlike the rest of the sequence, they exhibit $d_{NN}(i,i+1)$ and/or $d_{\alpha N}(i,i+2)$ NOEs. The lack of such NOEs at other places in the sequence (i.e., before the first PRP repeat or within the repeats themselves) may of course reflect the absence of NH protons for proline residues. However, inspection of the spectra shows that the corresponding $d_{\delta N}(i,i+1)$ and $d_{\alpha\delta}(i,i+2)$ NOEs for proline residues are also absent, which argues against a simple lack of detectable protons. An exception is there is a $d_{\delta N}(i,i+1)$ NOE for residues 5 and 6; however, this falls within a turn region characterized by $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+2)$ NOEs over residues 5−7. It appears that the PRP repeats may provide random coil "spacers" between regions containing local structure.

$^3J_{NH-\alpha H}$ Coupling Constants. Accurate coupling constants are often difficult to measure in 50% TFE/water owing to the generally broad line widths in this solvent mixture. In the case of drosocin, the DQF−COSY spectrum was of a rather poor quality and was not used for analysis of couplings. For the non-glycosylated derivative, the relatively broad line widths in the spectrum precluded the detection of any small (<5 Hz) couplings that may have been present.

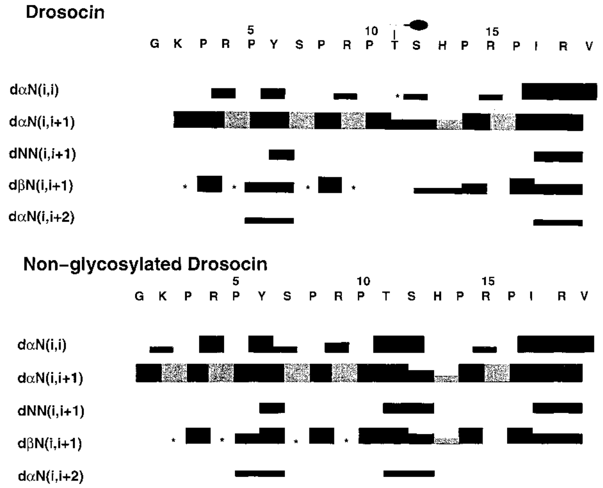

FIGURE 4: Summary of NOE connectivities in 50% TFE, 283 K, for (a, top) drosocin and (b, bottom) its non-glycosylated derivative. The sugar residues are represented by a half-shaded−half-solid "dumbbell" above the sequence. The intensities are indicated by the thickness of the line, grouped into strong, medium, and weak. Overlapping and therefore ambiguous cross-peaks are indicated by an asterisk. Connectivities that are not expected to be present due to the lack of the specified protons (i.e., Pro NH) are shaded.

However, large $^3J_{NH-\alpha H}$ coupling constants (>8.5 Hz), consistent with turns or local structured regions, were measured for residues Y6 and I17. These findings support the suggestions from the NOE data that turns occur near these residues. In particular, the combined coupling and NOE data strongly support the presence of a type I turn for residues 4−7, i.e., RPYS. A local structured region is also suggested near residue 17, although the data do not discriminate a particular type of turn. In addition to these structured elements common to both peptides, the NOE data are consistent with a type I turn at residues 10−13, PTSH, in the non-glycosylated peptide. While a large coupling constant in support of this proposal was not measured for S12, this may be due to extra broadening observed at this residue.

Hydrogen Bonding. Amide exchange experiments were conducted to determine whether there was any protection from solvent, indicative of hydrogen bonding. Both peptides were dissolved in 50% TFE/D_2O and the ^{1}H NMR spectra immediately examined for evidence of slow exchanging NHs. All of the NH signals disappeared rapidly and were completely gone after 0.5 h at room temperature. This indicated that the solution ensemble of conformers was not protected from solvent and that there were no long-lived, well-defined hydrogen bonds present.

Sugar−Peptide NOEs. Oligosaccharides attached to peptides are generally regarded as extremely flexible, and therefore it is interesting that a significant number of NOEs are observed between the peptide and sugar residues in drosocin. These NOEs are summarized in Figure 5. All of the NOEs are to the directly attached sugar (*N*-acetylgalactosamine) but, because there is spectral overlap of galactose shifts with peptide shifts, the possible presence of other NOEs to the second sugar cannot be completely excluded.

It is interesting that the observed NOEs include several from the peptide backbone amide protons (T11 and H13, but not S12), suggesting close proximity of the glycan unit to the peptide. The corresponding NOE to the amide proton of S12 is clearly absent; i.e., if present, this peak would occur in a nonoverlapped region of the spectrum, so its absence cannot be attributed to overlap. In an extended peptide backbone, the positions of sequentially adjacent amide protons are distant from one another, whereas in a type I turn they are close. So, the fact that NOEs are detected from GalNAc to the amide protons of T11 but not S12 supports the suggestion of a change from a type I turn to a more extended local conformation near these residues in drosocin. However, some degree of folded structure must be maintained because an NOE from the sugar to H13 is observed. The results are readily interpretable in terms of the opening of a type I turn to a more extended "bend" in the glycosylated form.

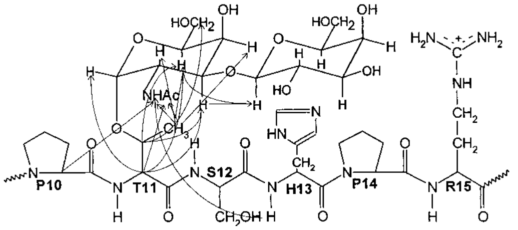

FIGURE 5: Schematic depiction of NOE connections observed between the peptide and glycan moieties of glycosylated drosocin.

The observed NOEs to the *N*-acetylgalactosamine residue are predominantly on one face of the sugar, further supporting the suggestion of a relatively immobilized linkage between the peptide and sugar. The observation of NOEs between amide and sugar protons in this small glycopeptide is remarkable. Firstly, NOEs for small peptides are generally weak, and secondly, the use of 50% deuterated TFE meant that only 50% of the available amide intensity was present. Thus, the results provide clear evidence of relatively long-lived interactions between the peptide backbone and the sugar moiety.

Histidine Titrations. In preliminary spectra of drosocin a variation in the line widths of the K2, S7, and H13 amide protons in the TOCSY spectra as a function of temperature and pH was noted. In particular, the histidine resonances seemed very sensitive to solution conditions. Since H13 is close to the glycosylation site (schematically illustrated in Figure 5) and an NOE was observed between H13 and the sugar residue, it was thought the sugar substitution might affect the pK_a of this residue. This, in turn, could perhaps explain the different biological activities exhibited by drosocin and its non-glycosylated derivative. Thus, two series of 1D, TOCSY, and NOESY spectra of the two peptides were recorded as a function of pH to detect if there were any effects on conformation and to determine the pK_a of H13.

In general, it was found there was little change in αH chemical shifts of any residue in either peptide over the pH range 3.5−7.5, indicating that pH does not have a significant influence on the conformation of either peptide. In contrast with the αH protons, the H2 and H4 aromatic residues of H13 underwent substantial changes in chemical shifts and line widths during the course of the titration. The chemical shift changes are illustrated in Figure 6. The derived pK_as for H13 were 5.5 and 5.6, in the glycosylated and non-glycosylated peptides, respectively. The values are indistinguishable within experimental error and indicate no specific change in pK_a induced by glycosylation. Both values are slightly less than the expected value for histidine in a random

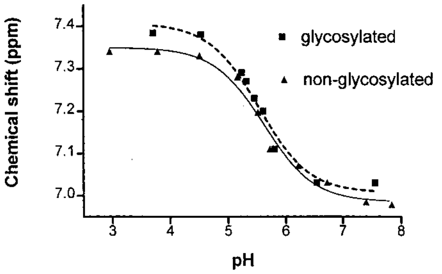

FIGURE 6: pH titration of drosocin and its non-glycosylated derivative.

coil peptide; however, this may reflect the influence of the TFE in the solution. Organic solvents are well-known to cause perturbations of this magnitude in apparent pK_a values.

Temperature Coefficients. The sensitivities of the amide proton chemical shifts to temperature were measured from a series of one-dimensional spectra of drosocin. Linear plots of the shifts versus temperature were obtained for all residues except K2, S7, and I17. For these residues, overlap was such that it was impossible to measure their chemical shifts accurately over the whole temperature range. All other residues had temperature coefficients between 6.2 and 10.7 ppb/K, suggesting a lack of intramolecular hydrogen bonds which would have indicated the presence of defined secondary structure. These data are consistent with the NOE and chemical shift data which suggest that drosocin and its non-glycosylated derivative are largely unstructured. The populations of locally structured forms, detected from the NOE data, are too small to be detected by the temperature coefficient data.

Structure Calculations. A total of 159 distance restraints consisting of 83 intraresidual, 71 sequential, and 5 medium-range NOEs, assigned from the 300 ms NOESY at 283 K, was used to generate a set of 100 structures. Analysis of these structures confirmed that drosocin has significant populations of essentially random coil conformations in aqueous solution. This may be gauged from the relatively poor root mean square deviation (>5 Å) for a superposition of 100 structures, but it is interesting that many of the 100 structures do confirm the presence of the turn elements identified in the earlier qualitative analysis of NOE data. A low-energy structure (Figure 7) illustrates the presence of the two turns involving residues 5−7 and 17−19 as well as an "bend" centered at the glycosylation site. It should be stressed again that drosocin does not have a single solution conformation, but the NOE data are consistent with a small population of solution conformers with the general features illustrated in Figure 7.

DISCUSSION

In this study the structures of an antibacterial peptide and its non-glycosylated derivative were examined to determine the possible consequences of sugar incorporation which is known to have a marked effect on the biological activity of the peptide.

The NMR data show that both peptides are unstructured in water and largely unstructured even in 50% TFE/water. However, in the latter solvent there are clear indications that within the population of unstructured forms there are some subpopulations which have elements of local structure. In particular, there appears to be a type I turn at residues 4−7 in both peptides and at 10−13 in the non-glycosylated form. Both peptides also exhibit some local structuring of a less well-defined nature at the C-terminus (residues 17−19).

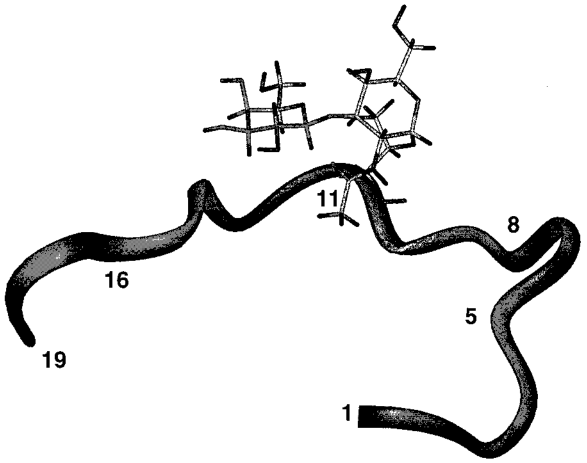

FIGURE 7: Representative structure from the simulated annealing and energy minimization calculations on drosocin. Shown clearly are the two turns at residues 5−7 and 17−19 as well as the open turn centered around the glycosylation site.

These findings are in agreement with a previous CD study (*3*) which found that in water the two peptides exhibited CD curves typical of peptides whose secondary structures have no conformational preferences (*50*) but, in 90% TFE, the CD spectra of the two peptides were consistent with the presence of β-turns (*51*, *52*). The NMR data significantly extend our understanding of the conformational features of drosocin by identifying the type and location of the β-turns surmised from the CD data. In addition, the NMR data clearly indicate a difference between the glycosylated and non-glycosylated forms, i.e., in the perturbation of a turn directly adjacent to the glycosylation site. In this regard drosocin is indeed a good model for studying the effect of sugar incorporation on the secondary structure of natural O-linked glycopeptides and glycoproteins.

It was concluded from the CD study that the conformations of both peptides were essentially the same. However, it was noted that, after glycosylation, a broad band at 203 nm became somewhat red-shifted and sharper, suggesting minor stabilization of turn structures. The latter observation is in agreement with other studies which have revealed that sugars are commonly associated with turns in peptides (*17*, *19*, *49*). From the NMR data, it appears the turn structure at residues 10−13 changes significantly on glycosylation. A loss of short-range NOEs suggests the turn opens out to a more extended structure, although the precise nature of the new local structure was not resolved. Some tightening of a turn near residues 17−19 is also detected from the NMR data, as demonstrated by an extra $d_{\alpha N}(i,i+2)$ NOE on glycosylation. The opening of one turn and the tightening of another may result in little net change in the amount of β-turn structure, thus explaining why little change was detected in CD spectra. This illustrates the advantages of a technique such as NMR which allows structural changes to be localized to specific parts of the peptide sequence.

The structural studies provide some insight into possible modes of action of drosocin. First, the data clearly show that the peptide does not adopt a helical conformation. Medium-range NOE connectivities suggestive of helical conformations

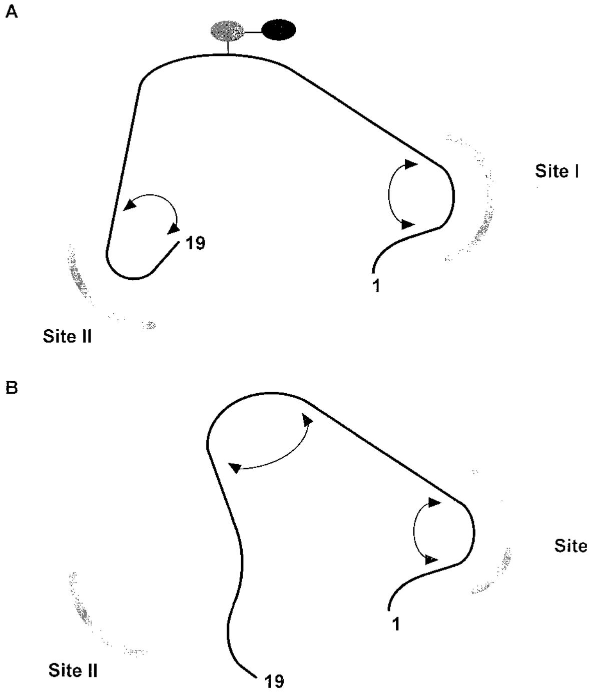

FIGURE 8: Schematic diagram showing the possible interaction of non-glycosylated and glycosylated drosocin with its receptor. One likely possibility is that the glycosylation of drosocin opens the turn at residues 11−13, thus aiding in the correct orientation of two separate binding regions centered around the 5−7 turn and 17−19 turn.

were notably absent in both peptides. In particular, there were no $d_{\alpha N}(i,i+3)$ connectivities, which are typical of α-helices. This is consistent with the activity data which showed that drosocin does not act as a pore-forming antimicrobial peptide. Pore-forming antimicrobial peptides often have amphipathic helices as at least part of their structure in order to facilitate their insertion into membranes (*53*).

Second, it is remarkable that all three regions of the peptide for which local structured elements were identified have been associated with perturbations of the activity of drosocin. Removal of the first five residues (*3*) or the last two residues (*7*) reduces activity. Similarly, deglycosylation mid-sequence at T11 reduces activity up to 8-fold. On the basis of these results, it is possible the interaction of drosocin with its receptor may involve the locally structured regions 4−7 and 17−19 in drosocin and that glycosylation may aid in correctly orienting these separate binding regions, as illustrated in Figure 8.

As already mentioned, drosocin belongs to the proline-rich family of insect antimicrobial peptides. This groups also includes apidaecin Ia, Ib, and II, abaecin, formaecin 1 and 2, pyrrhocoricin, lebocin I, II, and III, and diptericin. From the sequences of these shown in Figure 9, it is apparent there is partial conservation of the PRP sequence which was found in the current study to precede regions of local folded structure. Of the peptides shown, formaecin 1 and 2, pyrrhocoricin, the lebocins, and diptericin also carry an O-glycosylated substitution. Interestingly, structure−activity studies on apidaecin and pyrrhocoricin reveal a similarity between these peptides and drosocin. Both apidaecin's and pyrrhocoricin's antibacterial activities are primarily directed toward gram-negative bacteria, the D-stereoisomer of apidaecin is inactive, and both peptides require an incubation period of 24 h with bacteria to be effective (*2*, *54*). At present, the exact role the sugar substitution plays in drosocin's antibacterial activity remains unknown. A recent study on the activity and metabolic stability of synthetic pyrrhocoricin, drosocin, and their analogues tends to suggest the sugar does not have a primary role in killing bacteria. Results of this study showed that while addition of the Gal-GalNAc disaccharide increased the in vitro biological activity of drosocin, glycosylation of the homologous pyrrhocoricin caused a decrease in antimicrobial potency. Also, despite the similar amino acid and sugar composition, the two glycopeptides underwent different degradation pathways in mammalian sera. This indicates differences in the accessibility of the terminal regions and conformations between the two peptides. These observations suggest the disaccharide moiety in drosocin is not directly involved in the antimicrobial activity but is involved in secondary effects such as the proper orientation of the receptor binding domains (*7*). While the carbohydrate appears to help drosocin assume the right secondary structure by slightly modifying a mid-sequence turn, a similar minor alteration in pyrrhocoricin may negatively influence the presentation of the peptide to its stereospecific target. High-resolution conformational analysis of non-glycosylated and glycosylated pyrrhocoricin may substantiate this hypothesis.

Drosocin	--GK**PRP**YS**PRP**TSH**PRP**IRV
Formaecin 1	--GRPNPVNNKPTPYPHL
Formaecin 2	--GRPNPVNTKPTPYPRL
Pyrrhocoricin	-VDKGS-YL**PRP**T-P**PRP**IYNRN
Diptericin	-DEKPK--LILPT--PAPPNLPQ.............
Apidaecin Ia	GNNRP-VYIPQ**P-RP**PHPRI
Apidaecin Ib	GNNRP-VYIPQ**P-RP**PHPRL
Apidaecin II	GNNRP-IYIPQ**P-RP**PHPRL
Abaecin	YVPLPNV--PQPGRRPFPTFPGQ-GPFNPK-IKWPQGY
Lebocin I and II	--DLRFL--YPRGKLPVPTPP----PFNPKPIYIDMGNRY

FIGURE 9: Comparison of sequences from formaecin 1 and 2 (*Myrmecia gulosa*), pyrrhocoricin (Pyrrhocoris apterus), N-terminal region of diptericin (*Phormia terranovae*), apidaecin Ia, Ib, and II (*Apis mellifera*), abaecin (*Apis mellifera*), and lebocin I, II, and III (*Bombyx mori*) with drosocin.

The major role of glycosylation appears to be orienting the two terminal turns via an effect on a central turn; however, it also seems there may be some strengthening of the C-terminal turn via glycosylation. No such strengthening effect is seen at the (already strong) N-terminal turn. This is very interesting as two recent studies have reported long-range turn-stabilizing effects of O-glycosylated sugars on two peptides from the V3 loop of HIV-1_{IIIB}. In the first study it was found that, upon glycosylation of the 15-residue peptide, a turn upstream of the site appeared to be tightened or stabilized. It was suggested that perhaps the sugar, by restricting the local conformation of the peptide, was also able to transmit an effect to more distant regions of the peptide (*55*). In the second study a longer fragment (24 residues) of the V3 loop, which incorporated an additional O-glycosylation site upstream of the turn, was investigated. This study confirmed the results of the first. In addition, it also revealed that the presence of this second sugar appeared to tighten or stabilize the turn to a greater extent than the sole presence of one sugar downstream of the turn in the 15-residue peptide (*56*). Both of these studies suggest that perhaps the sugars, by restricting the local conformation of the peptides, are able to trasnmit an effect to more distant regions of the peptides. This is what has been observed with drosocin.

The V3 studies also reported that, like drosocin, the sugars have a defined conformation with respect to the peptide backbones (*55*, *56*). For drosocin the evidence for this is derived from the large number of NOEs between the sugar and the peptide. Most of these NOEs are between T11 and the GalNAc residue. However, there is also a weak NOE between the backbone amide proton of H13 and the acetamido group of GalNAc. There is also a significant change in the amide chemical shift of R15 but not of other nonproximate residues. The downfield shift in the presence of the sugar suggests there may be a hydrogen-bonding interaction of the side chain amide of R15 with the disaccharide.

In summary, this study has shown that drosocin and its non-glycosylated derivative exist largely as random coil conformations in solution, but there are small populations of structured forms within the solution ensemble. There are a number of differences in NOE and chemical shift patterns between the two peptides, and these indicate a conformational change associated with this small population of structured forms. It is interesting to note NMR tends to suggest that rather than a single effect of stabilizing a specific turn within the peptide, glycosylation actually results in a shift toward a more extended conformation around the glycosylation site and generates a new set of different turn variants at other parts of the molecule.

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