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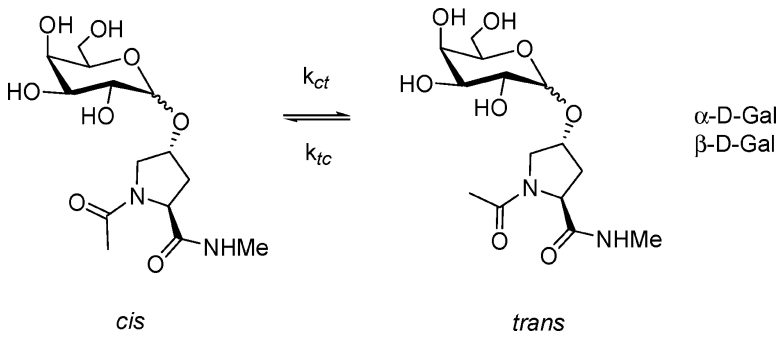

cis *trans*

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Effects of Glycosylation of (2*S*,4*R*)-4-Hydroxyproline on the Conformation, Kinetics, and Thermodynamics of Prolyl Amide Isomerization

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Glycosylation is a common post-translational modification of proteins implicated in cellular recognition processes and controlling protein conformation.[1] Typically, carbohydrates are *O*-linked to serine (Ser) and threonine (Thr) or *N*-linked to asparagine (Asn). Glycosylation of (2*S*,4*R*)-4-hydroxyproline (Hyp) is widespread in the plant kingdom and occurs in Hyp-rich glycoproteins (HRGPs) that are associated with the cell walls of algae and flowering plants.[2] HRGPs are characterized by extensively glycosylated Hyp sequences that contain *O*-glycosidic linkages to the pyranose D-galactose or the furanose L-arabinose.[2] Although HRGPs are broadly implicated in many aspects of plant growth, development[3] and cell wall stability,[2] no information is available about the structural and conformational implications of Hyp-glycosylation on peptide backbone conformation.

Hyp and proline (Pro) are unique among the proteinogenic amino acids since they are characterized by limited rotation of the ø dihedral angle (Figure 1) as their side chain is fused to the peptide backbone. As a consequence, there is a reduction in the energy difference between the prolyl amide *cis* and *trans* isomers, making them nearly isoenergetic; this leads to higher *cis N*-terminal amide content relative to the other amino acids. Moreover, the isomerization of the prolyl amide bond has been shown to be the rate-determining step in the folding pathways of many peptides and proteins.[4]

Herein we describe the effects of galactosylation of Hyp on the conformation as well as the thermodynamics and kinetics of prolyl *N*-terminal amide isomerization. Compounds **4a** AcHyp(α-D-Gal)-NHMe and **4b** AcHyp(*β*-D-Gal)NHMe were selected as glycopeptide mimics, while AcProNHMe **1**, AcHypNHMe **2**, and AcHyp(O-*tert*-butyl)NHMe **3** served as non-glycosylated reference compounds (Figure 1). The *trans* rotamers in compounds **1**−**4b** were assigned on the basis of higher C^{δ} atom NMR chemical shifts relative to the *cis* rotamer[5] and nOe transfer between H-*δ* of proline and the *N*-acyl protons in selective 1D GOESY experiments.[6] The relative amounts of *cis* and *trans* isomers were determined by integrating and averaging as many distinct proton signals as possible for both the major and minor isomers in the ^{1}H NMR spectra.[7] In D_2O at 37 °C, the *trans*/*cis* isomer ratio equilibrium constant ($K_{t/c}$) for **4a** (3.41 ± 0.30) and **4b** (3.37 ± 0.28) are nearly identical to those of **2** (3.52 ± 0.05) and **3** (3.34 ± 0.15), and the observed differences are within the experimental errors (Table 3).

The kinetics of *cis*/*trans* isomerization for compounds **1**−**4b** were determined by ^{1}H NMR spectroscopy inversion transfer experiments[8] in D_2O at elevated temperature. At 67 °C, the *cis*-to-*trans* rate constant of isomerization (k_{ct}) of the α-glycosylated Hyp model peptide **4a** (k_{ct} = 0.83 s^{-1}) is very similar compared to the hydroxyproline model peptide **2** (k_{ct} of 0.73 s^{-1}) and **3** (k_{ct} = 0.77 s^{-1}), while the *β*-anomer **4b** gave slightly lower rates (k_{ct} = 0.61 s^{-1}). A similar trend was observed for the *trans*-to-*cis* rate constants of compounds **1**−**4b** (Table 1). At physiological temperature the kinetic rates are too slow to be differentiated by this assay.

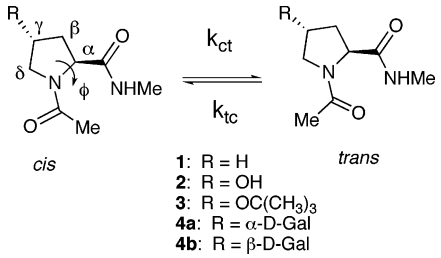

Figure 1. *Cis-trans* isomerization of reference diamides **1**−**3** and the galactosylated Hyp model amides **4a** and **4b**.

Table 1. Rates of Prolyl Amide Isomerization for **1**−**4b**

amide	k_{ct}[a] (s^{-1})	k_{tc}[b] (s^{-1})
1	0.81 ± 0.02	0.31 ± 0.02
2	0.73 ± 0.01	0.25 ± 0.01
3	0.77 ± 0.02	0.27 ± 0.01
4a	0.83 ± 0.05 (0.82 ± 0.06)[c]	0.27 ± 0.02 (0.27 ± 0.03)[c]
4b	0.61 ± 0.04 (0.57 ± 0.05)[c]	0.21 ± 0.02 (0.19 ± 0.02)[c]

[a] Carried out in D_2O at 67 °C. [b] Calculated from k_{ct} and equilibrium ($K_{t/c}$). [c] Carried out in phosphate buffer pH = 7.4 at 67 °C.

Table 2. Activation Enthalpies ($\Delta H^{\ddagger}$) and Entropies ($\Delta S^{\ddagger}$) as Derived from Eyring Plots in D_2O for **1**−**4b**

	cis to *trans*[a]			*trans* to *cis*[a]		
amide	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ cal/mol·K	$\Delta G^{\ddagger}_{300K}$ kcal/mol	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ cal/mol·K	$\Delta G^{\ddagger}_{300K}$ kcal/mol
1	20.6	1.2	20.2	21.3	1.4	20.9
2	20.2	0.1	20.2	21.1	0.5	21.0
3	21.8	4.7	20.4	22.7	5.4	21.1
4a	20.4	0.6	20.2	20.6	1.0	20.3
4b	22.4	6.2	20.6	23.3	6.6	21.3

[a] Error limits obtained from the residuals of the linear least-squares fitting of the data to equation $\ln(k/T) = (-\Delta H^{\ddagger}/R)(1/T) + \Delta S^{\ddagger}/R + \ln(k_B/h)$ were 1−2% for compounds **1** and **2**, and 3−6% for compounds **3**, **4a**, and **4b**.

Table 3. Thermodynamic Parameters for Isomerization of **1**-**4b**

Amide	$\Delta H°$ [a] (kcal/mol)	$\Delta S°$ [a] (cal/mol·K)	$K_{t/c}$[b] (37 °C)
1	−0.95 ± 0.01	−0.87 ± 0.02	3.03 ± 0.08
2	−1.33 ± 0.03	−1.76 ± 0.09	3.52 ± 0.05
3	−1.29 ± 0.02	−1.72 ± 0.06	3.34 ± 0.15
4a	−1.27 ± 0.02	−1.64 ± 0.07	3.41 ± 0.30
4b	−1.30 ± 0.03	−1.77 ± 0.10	3.37 ± 0.28

[a] Error limits obtained by linear least-squares fitting the data of the van't Hoff plots to equation $\ln K_{t/c} = (-\Delta H°/R)(1/T) + \Delta S°/R$; [b]Carried out in D_2O; ± SE determined by integration of two or more sets of *trans/cis* isomers.

The effects of temperature on k_{ct} and k_{tc} were analyzed by Eyring plots[9] (Supporting Information) and values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were calculated from linear least-squares fits of the data in these plots and are presented in Table **2**. The activation parameters demonstrate that the free-energy barriers to isomerization of compounds **1**−**4b**

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are enthalpic in origin. The effects of temperature on the values of $K_{t/c} = (k_{ct}/k_{tc})$ were measured directly by NMR spectroscopy, and the resulting data were analyzed by van't Hoff plots (Supporting Information). Values for $\Delta H°$ and $\Delta S°$ were calculated from linear least-squares fits of these plots (Table 3). In all cases studied, the *trans* isomer of **1**−**4b** is more stable than the *cis* isomer. Moreover, the values of $K_{t/c}$ for **1**−**4b** are dependent on temperature such that the *trans* isomer becomes increasingly favored as the temperature decreases.

The pucker of Hyp (2*S*,4*R*) in model peptide **2** in solution has been previously assigned to the Cγ-exo conformation on the basis of observed J-coupling constants.[10] The prolyl ring pucker for compounds **3**, **4a**, and **4b** were similarly established as the Cγ-exo conformation on the basis of ^{1}H NMR coupling constants by comparison to literature values. For example, in compound **4a** we observed both $^3J_{\alpha\beta1}$ and $^3J_{\alpha\beta2}$ couplings constants to be 8.2 Hz. The expected coupling constants for the Cγ-exo conformer are 7−10 Hz and 7−11 Hz, respectively, whereas those for Cγ-endo are 6−10 Hz and 2−3 Hz, respectively. Similarly, other couplings show characteristic patterns for the Cγ-exo pucker (Supporting Information).

Previous reports have shown that inductive effects in the γ-position of proline have significant structural consequences on the thermodynamics and kinetics of prolyl amide bond isomerization.[11] To assess the inductive effect caused by glycosylation of hydroxyproline we determined the ^{13}C NMR chemical shifts of the C$^\gamma$ atom, which can indicate electron withdrawal by pendant function groups,[12] and have previously been used to correlate the electron-withdrawing effects in various C$^\gamma$-substituted proline analogues.[11c] The observed ^{13}C NMR chemical shifts ($\delta_{C\gamma(trans)}$) indicate that electron withdrawal increases in the order hydroxyl ($\delta_{C\gamma} = 69.9$) (**2**) < *tert*-butoxyl ($\delta_{C\gamma} = 70.1$) (**3**) < α-galactosyl ($\delta_{C\gamma} = 76.5$) (**4a**) < β-galactosyl ($\delta_{C\gamma} = 77.9$) (**4b**) (see Table 1 in the Supporting Information).

To determine the extent of interaction between the galactose and prolyl rings, we performed nOe transfer experiments with compounds **4a** and **4b** in D_2O. Selective inversion of one of the H-β protons in **4a** by a selective GOESY[6] experiment resulted in a 1.5% resonance transfer to a peak at $\delta = 3.83$ ppm corresponding to the overlapped signals of both H-4 and H-5 of galactose. By comparison, selective inversion of H-2 in **4b** produced nOe transfer (3.0%) to H-α of Hyp. These results suggest that galactosylation of Hyp induces close contacts between distant positions in the carbohydrate and pyrrolidine rings.

In summary, we have found that glycosylation of Hyp in compounds **4a** and **4b** has no apparent effect on the isomer equilibrium ($K_{t/c}$) nor on the rate of isomerization (k_{tc}, k_{tc}) in water between the *cis* and *trans* isomers when compared to unglycosylated reference compounds **1**−**3**. However, glycosylation of Hyp provides an inductive electron withdrawing effect on the prolyl ring. The magnitude of the change in ^{13}C NMR chemical shift (6.5−8 ppm) has been correlated to the strength of the inductive effect and is similar to a downfield shift of 8 ppm observed when a trifluoroacetate group was attached to Hyp in AcHypOMe.[11c] The effect is slightly larger for AcHyp(β-D-Gal)NHMe **4b** (by 1.5 ppm) compared to the α-anomer **4a**. It is known that (4*R*)-electronegative substituents stabilize the Cγ-exo pucker of proline in peptide mimics[11] and contribute to enhanced stability of the triple helix in collagen.[13] Moreover, it has been established both from computational[14] and experimental studies[15] that stabilization of the Cγ-exo ring pucker favors the prolyl *trans* amide isomer. Our results therefore suggest that glycosylation of Hyp may lead to additional stabilization of the Cγ-exo ring pucker of Hyp. However, this stabilization does not translate in a measurable increase on $K_{t/c}$ for peptide mimics **4a** and **4b** when compared to unglycosylated **2**. Most likely the stabilization of the *trans* isomer in compounds **4a** and **4b** is too small to be differentiated and remains within the experimental error. Any stabilization of the Cγ-exo ring pucker may be more apparent in larger glycopeptides that contain two or more glycosylated Hyp units, where the effect may be additive. Perhaps more importantly, nOe experiments show that the glycosylation of **2** results in distant contacts between the proline and galactose rings, suggesting that galactosylation of Hyp induces conformational constraint into glycopeptides. Very likely this is the result of increased steric strain induced upon glycosylation. Additionally, glycosylation of Hyp may also affect other properties including solvation, solubility, and thermostability. In conclusion, while there is no significant influence on prolyl *N*-terminal amide isomerization, the presence of both an enhanced inductive effect and Gal-Pro contacts between distant positions in **4a** and **4b** suggests that glycosylation of Hyp will have important implications on peptide backbone conformation in HRGPs and glycosylated Hyp-containing peptides.

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Supporting Information Available: Synthetic procedures, ^{1}H NMR, ^{13}C NMR, 1D-GOESY spectra, Eyring plots, van't Hoff plots, plots of intensity versus mixing time for the magnetization transfer experiments of **1**−**4b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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