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The Implications of (2*S*,4*S*)-Hydroxyproline 4-*O*-Glycosylation for Prolyl Amide Isomerization

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Abstract: The conformations of peptides and proteins are often influenced by glycans *O*-linked to serine (Ser) or threonine (Thr). (2*S*,4*R*)-4-Hydroxyproline (Hyp), together with L-proline (Pro), are interesting targets for *O*-glycosylation because they have a unique influence on peptide and protein conformation. In previous work we found that glycosylation of Hyp does not affect the *N*-terminal amide *trans*/*cis* ratios ($K_{trans/cis}$) or the rates of amide isomerization in model amides. The stereoisomer of Hyp—(2*S*,4*S*)-4-hydroxyproline (hyp)—is rarely found in nature, and has a different influence both on the conformation of the pyrrolidine ring and on $K_{trans/cis}$. Glycans attached to hyp would be expected to be projected from the opposite face of the prolyl side chain relative to Hyp; the impact this would have on $K_{trans/cis}$ was unknown. Measurements of ^3J coupling constants indicate that the glycan has little impact on the C$^\gamma$-*endo* conformation produced by hyp. As a result, it was found that the D-galactose residue extending from a C$^\gamma$-*endo* pucker affects both $K_{trans/cis}$ and the rate of isomerization, which is not found to occur when it is projected from a C$^\gamma$-*exo* pucker; this reflects the different environments delineated by the proline side chain. The enthalpic contributions to the stabilization of the *trans* amide isomer may be due to disruption of intramolecular interactions present in hyp; the change in enthalpy is balanced by a decrease in entropy incurred upon glycosylation. Because the different stereoisomers—Hyp and hyp—project the *O*-linked carbohydrates in opposite spatial orientations, these glycosylated amino acids may be useful for understanding of how the projection of a glycan from the peptide or protein backbone exerts its influence.

Keywords: carbohydrates · glycoconjugates · glycopeptides · peptide glycosylation

Introduction

Glycosylation is a common post-translational modification of proteins in eukaryotes, as well as in bacteria and archaea.[1] Aside from direct involvement in various biological processes involving protein–carbohydrate recognition,[2] glycosylation also affects protein conformation and folding,[2a,b] receptor binding and signalling,[2a,b] enhancement of the thermal stabilities of proteins,[3] protection against proteolytic degradation,[4] hydration and hydrophilicity,[5] and may also facilitate membrane penetration.[6]

In order to explore the effects of glycosylation on peptide backbone conformation, many small-model glycopeptides and glycopeptide mimics have been prepared over the years.[7] These studies have concluded that the nature of the glycosidic linkage not only influences the presentation of the carbohydrate moiety, but also influences peptide backbone conformation.[2d,8] Typically, carbohydrates are *O*-linked to serine (Ser) and threonine (Thr) or *N*-linked to asparagine (Asn). *O*-Glycosylation of (2*S*,4*R*)-4-hydroxyproline (Hyp) is widespread in the plant kingdom and occurs in hydroxyproline-rich glycoproteins (HRGPs) that are associated with the cell walls of algae and flowering plants.[9] The stereoisomer of Hyp—(2*S*,4*S*)-4-hydroxyproline (hyp)—is rarely found in nature, but has been isolated from extracts of the sandalwood tree *Santalum album*, several species of fungi and the cyanobacteria *Lyngbya majuscula*.[10]

Proline (Pro), Hyp and hyp exhibit properties unique among proteinogenic amino acids. Firstly, these amino acids are characterized by limited rotation of their ϕ dihedral angles, because their side chains are fused to the peptide backbones. As a result, there is a reduction in the energy difference between the prolyl amide *cis* ($\omega = 0°$) and *trans* ($\omega = 180°$) isomers, making them nearly isoenergetic

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(Figure 1);[11] this leads to higher *cis* *N*-terminal amide isomer content than in the case of the other amino acids. Secondly, prolyl *cis*/*trans* isomerization is often the rate-determining step in the folding pathways of many peptides and proteins,[11a,12] and thirdly, Pro and Hyp induce β turns and extended helical structures (polyproline helixes), crucial in protein–protein and protein–peptide interactions, in peptides.[13,14] Moreover, both Pro and Hyp play important roles in the stabilities of structural proteins such as collagen[15] and have also been implicated in contributing to the stabilities of HRGPs such as the extensins.[16] As a result, there is growing interest in understanding and controlling prolyl *N*-terminal amide isomerization in biological processes.

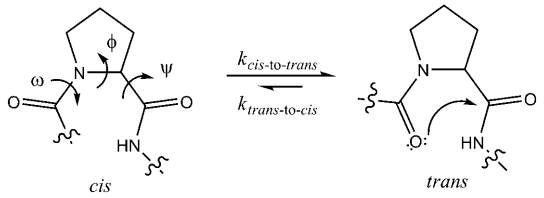


Figure 1. Proline *cis* amide isomers showing relevant backbone torsion angles as well as the *trans* amide isomer with the n → π* interaction between the *N*-terminal amide carbonyl oxygen and the *C*-terminal carbonyl carbon.

The prolyl *cis*/*trans* isomerization equilibrium is mostly governed by an n → π* interaction between the oxygen lone pair from the prolyl *N*-terminal amide C=O and the antibonding orbital of the *C*-terminal C=O (Figure 1).[11,17–20] Recent studies have shown that 4-hydroxylation and attachment of other electron-withdrawing groups have a further influence on *cis*/*trans* isomerization through inductive and stereoelectronic effects,[17,21–25] which affect the n → π* interaction through changes to the conformation of the pyrrolidine ring[26,27] and the prolyl backbone ψ dihedral angle.[17,21–25] It has also been shown that carbohydrates can affect *cis*/*trans* isomerization, because Ser *O*-glycosylated either with α-linked *N*-acetylgalactosamine or with β-linked *N*-acetylglucosamine *N*-terminal to Pro stabilizes the *trans* amide conformation.[28] Recently, our group has developed several unnaturally *C*-glycosylated Pro analogues, which have demonstrated strong abilities to vary the prolyl *N*-terminal amide equilibrium ($K_{trans/cis}$).[29]

In terms of clarifying the effects of 4-*O*-glycosylation of Hyp, as found in HRGPs, we demonstrated in earlier work that neither α- or β-glycosylation of Hyp in model amides had any apparent effect either on the isomer equilibrium constants or on the rates of amide isomerization.[30] However, our results demonstrated that galactosylation of Hyp provides an inductive electron-withdrawing effect on the prolyl ring. It is known that (4*R*)-electronegative substituents stabilize the C^{γ}-*exo* pucker of proline.[17,21–25] Moreover, NOE experiments indicated that glycosylation of Hyp resulted in distant contacts between the proline and galactose rings, which indicates that glycosylation induces conformational constraint into glycopeptides. From these results, we became interested in studying how galactosylation of hyp, the stereoisomer of Hyp, would influence the thermodynamics and kinetics of prolyl amide *cis*/*trans* isomerization. We anticipated that *O*-glycosylation of hyp might have a different impact on *N*-terminal amide isomerization, because 4*S*-hydroxylation would cause the hydroxy group to be projected from the opposite face of the prolyl side chain relative to its stereoisomer (Figure 2). Whereas Hyp has been found to adopt a C^{γ}-*exo* conformation, hyp is associated with a C^{γ}-*endo* conformation;[31–35] the closely related C^{β}-*exo* conformation has also been suggested.[36,37] This conformational switch has been attributed to a stereoelectronic effect, through which the 4-hydroxy group prefers to adopt a *gauche* orientation with the prolyl nitrogen atom.[17,21–25] This orientation is further stabilized by hyperconjugative $\sigma(C^{\beta}\text{–H}) \rightarrow \sigma^*(C^{\gamma}\text{–O})$ and $\sigma(C^{\delta}\text{–H}) \rightarrow \sigma^*(C^{\gamma}\text{–O})$ interactions. As a result, Hyp favours the *trans* amide conformation relative to Pro because the C^{γ}-*exo* pucker forces a ψ angle of 150°, which is ideal for a favourable n → π* interaction; this interaction between the *N*-terminal amide carbonyl oxygen and the *C*-terminal carbonyl carbon has been shown to stabilize the *trans* amide (Figure 1).[17,21–25] In contrast, the C^{γ}-*endo* conformation associated with hyp has been shown to favour the *cis* amide conformation because of an unfavourable ψ dihedral angle for the same n → π* interaction, which has been attributed to several factors: experimental and computational methods indicate that in hyp a hydrogen bond probably exists between the 4-hydroxy group and the *C*-terminal carbonyl oxygen atom, as well as electrostatic repulsion between the oxygen atom in the 4-position and the *C*-terminal carbonyl oxygen atom.[17,35] Both of these factors are likely to force the prolyl ψ angle into a poor orientation for the n → π* interaction, resulting in hyp favouring the *cis* amide conformation more than Pro and Hyp.

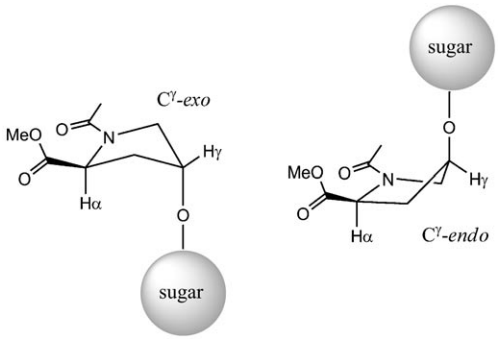


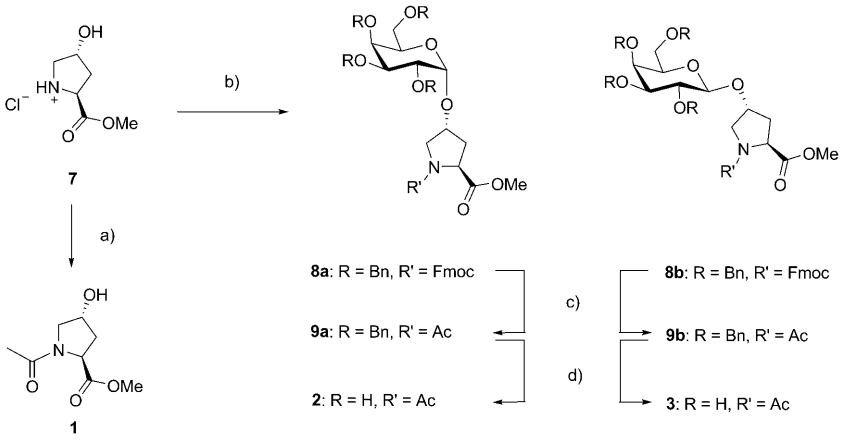
Figure 2. The C^{γ}-*exo* pucker of Hyp would place the sugar below the plane of the proline ring, whereas the C^{γ}-*endo* pucker of hyp would place the sugar above the plane of the proline side chain.

Because glycosylation of hyp effectively places the carbohydrate moiety on a different face of the proline side chain than in the case of Hyp, it may have a different impact on proline isomerization. We explored the effects of glycosyla-

tion of (2*S*,4*S*)-4-hydroxyproline on prolyl conformation, and the kinetics and thermodynamics of prolyl amide isomerization, by using the monosaccharide D-galactose attached in both α- and β-anomeric configurations. Galactose was selected because of the occurrence of Hyp-Gal linkages in HRGPs.[9,16]

Results and Discussion

Synthesis: Model peptides of the *N*-acetyl-Pro methyl ester form are well established for the study of subtle effects of proline side chain modification on *N*-terminal amide isomerization.[17,21–25,26,38] This prevents the intramolecular hydrogen bonding and γ turn formation observed in *N*-acetylproline *N*′-methylamide model peptides.[39] Therefore, the model amides Ac-Hyp-OMe (**1**), Ac-[Hyp(α-D-Gal)]-OMe (**2**), Ac-[Hyp(β-D-Gal)]-OMe (**3**), Ac-hyp-OMe (**4**), Ac-[hyp(α-D-Gal)]-OMe (**5**) and Ac-[hyp(β-D-Gal)]-OMe (**6**) were synthesized by different strategies, depending on the commercial availability of each proline derivative (Schemes 1 and 2, below). We found that installation of the *N*-Fmoc group allowed optimal separation of the α- and β-anomers **8a**/**8b** (Scheme 1) and **11a**/**11b** (Scheme 2, below). Model amides containing both α- and β-anomeric linkages were made for comparison in order to explore how the nature of the glycosidic linkage affects prolyl amide *cis*/*trans* isomerization.

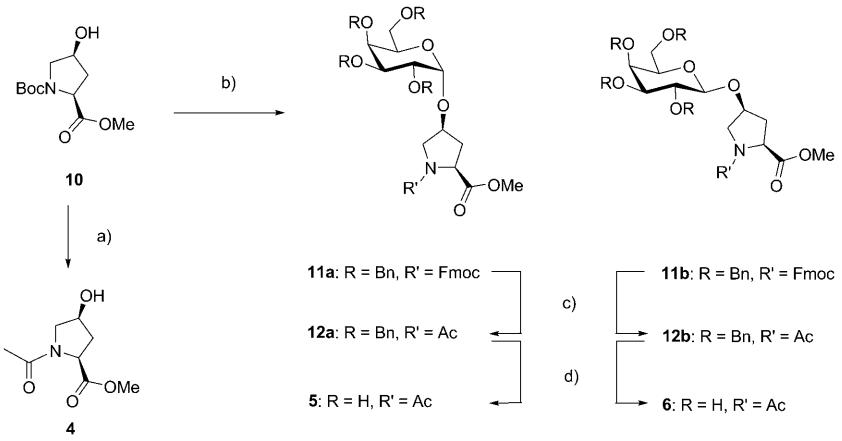


Scheme 1. Synthesis of **1**, **2**, and **3**. a) Ac_2O, Et_3N, MeOH, 25 °C, 2 h, 95 %; b) i) Fmoc-OPfp, $NaHCO_3$, acetone/H_2O 3:1, 25 °C, 3 h, ii) benzyl 1-thio-2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranoside, AgOTf, NIS, CH_3CN, 0 to 25 °C, 2 h, 35 % overall yield; c) i) CH_2Cl_2/piperidine 1:1, 25 °C, 3 h, ii) Ac_2O, Py, 25 °C, 4 h, 78 % overall yield; d) H_2/$Pd(OH)_2$/C, MeOH/ethyl acetate 4:1, 25 °C, 4 h, quantitative.

The synthesis of **1** was carried out by selective *N*-acylation of **7** with acetic anhydride and triethylamine in methanol in 95 % yield (Scheme 1). The glycosylated (4*R*)-hydroxyproline model peptides **2** and **3** were obtained through *N*-Fmoc protection of **7** (9-fluorenylmethyl pentafluorophenyl carbonate) under mild basic conditions, followed by glycosylation with benzyl 1-thio-2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranoside[40] in the presence of *N*-iodosuccinimide and catalytic amounts of silver triflate to yield both the α-linked (**8a**) and the β-linked (**8b**) products in 35 % yield over two steps. A 1.9:1 ratio of the α- and β-anomers was obtained under these conditions. Installation of the *N*-acetyl group was carried out by *N*-Fmoc removal (piperidine in dichloromethane) followed by *N*-acylation (acetic anhydride in pyridine) to give **9a** and **9b** in 78 % yield. Removal of the benzyl ether protecting groups by catalytic hydrogenolysis in methanol gave the model amides **2** and **3** in 27 % overall yield from **7**. Assignment of the α- and β-anomers was carried out by measuring the ^1H NMR $^3J_{H_1,H_2}$ coupling constant, which for **2** was 1.9 Hz, indicative of the *gauche* dihedral angle expected for the α-anomer, whereas $^3J_{H_1,H_2}$ for **3** was 8.0 Hz, indicative of the *trans* diaxial relationship expected for the β-anomer.

The synthesis of the (4*S*) model amides was carried out in a similar fashion beginning from **10** (Scheme 2). *N*-Boc removal (trifluoroacetic acid in dichloromethane at 0 °C) gave **4** in 94 % yield after *N*-acylation under standard conditions. Glycosylation of **10** was carried out under the same conditions as for **7**. It was found that separation of the α- and β-glycosides required replacement of the *N*-Boc group with the *N*-Fmoc protecting group, which was carried out under standard conditions to give **11a** and **11b** in 32 % yield over three steps, with a 1.5:1 ratio of the α- and β-anomers. Installation of the *N*-acetyl group to give **12a** and **12b**, followed by removal of the benzyl ether protecting groups, was carried out under the same conditions as for **8a** and **8b**, to give the model peptides **5** and **6** in 25 % overall yield over three steps.

IR spectroscopic study: The frequency of the amide I vibrational mode (ν_{amide}), which is primarily a function of the amide C=O stretching vibration, has been correlated with changes in the bond order of the amide C=O group.[41] In D_2O, the ν_{amide} values for the model compounds **1**, **2** and **3** were nearly identical, with maxima at 1612, 1611.5 and 1611.5 cm^{-1}, respectively. Similarly, nearly identical maxima of 1612, 1611 and 1613 cm^{-1} were exhibited by **4**, **5** and **6**, respectively. Therefore, for compounds **1**–**6**, no change in the amide carbonyl I vibrational mode was found to occur with α- or β-glycosylation or inversion of stereochemistry at the 4-position. A slightly more pronounced effect was observed in a study of 4-fluoroproline (Flp) in Ac-Flp-OMe model compounds, which



Scheme 2. Synthesis of **4**, **5**, and **6**. a) i) TFA/CH_2Cl_2 1:1, 0 °C, 1.5 h. ii) Ac_2O, Et_3N, MeOH, 25 °C, 2 h, 94 % overall yield; b) i) benzyl 1-thio-2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranoside, AgOTf, NIS, CH_3CN, 0 to 25 °C, 2.5 h, ii) TFA/CH_2Cl_2 1:1, 0 °C, 1.5 h, iii) Fmoc-OPfp, $NaHCO_3$, acetone/H_2O 3:1, 25 °C, 2 h, 32 % overall yield; c) i) CH_2Cl_2/piperidine 1:1, 25 °C, 3 h, ii) Ac_2O, Py, 25 °C, 4 h, 78 % overall yield; d) H_2/$Pd(OH)_2$/C, MeOH/ethyl acetate 4:1, 25 °C, 4 h, quantitative.

showed a maximum ν_{amide} shift—relative to (4*R*)-hydroxyproline in similar model compounds—of 3 cm^{-1} in D_2O.[21]

NMR spectroscopic studies: Full assignment of the ^1H NMR spectra of **1**–**6** was carried out with the aid of COSY and HSQC experiments. Assignment of the major isomers of **1**–**6** as the *trans* amide isomers in each case was established by selective one-dimensional GOESY[42] experiments, which showed interproton effects from the *N*-acetyl methyl group to the prolyl δ-protons (1.1–2.4 % NOE relative to the *N*-acetate singlet signal). By comparison, this interaction was not observed in the minor isomer.

Prolyl side-chain conformation: The prolyl ring puckers of the major isomers of **1**–**6** were established by comparison of ^1H NMR coupling constants with literature values (see the Supporting Information).[43] The prolyl puckers for **1**–**3** were each assigned as C^γ-*exo* on the basis of $^3J_{\alpha,\beta_1}$ values of 8.2–8.3 Hz and $^3J_{\alpha,\beta_2}$ values of 8.5–8.7 Hz. The corresponding coupling constants for the C^γ-*exo* pucker are expected to be 7–10 and 7–11 Hz, respectively. In contrast, the prolyl puckers for **4**–**6** were each assigned as C^γ-*endo* on the basis of $^3J_{\alpha,\beta_1}$ values of 7.8–9.8 Hz and $^3J_{\alpha,\beta_2}$ values of 2.4–4.3 Hz. The coupling constants for the C^γ-*endo* pucker are expected to be 6–10 and 2–3 Hz, respectively. These results are as expected in the context of other experimental[17,21–25,31–33,43] measurements and computational[34,44–46] predictions. The coupling constants for **4** and **6** were nearly identical at 25 °C, whereas those of **5** deviated slightly.

C^γ inductive effect: Changes in ^{13}C chemical shifts have been used to estimate the electron-withdrawing effects of substituents on the prolyl side-chain.[47] Therefore, measurement of the C^γ-carbon chemical shifts in the model compounds **1**–**6** was used to assess the relative changes in electron-withdrawing ability incurred upon glycosylation. Significant changes in the shift of the C^γ-carbon (~9 ppm) were found to occur upon glycosylation both for the (4*R*) model compounds (78.9, 77.6 ppm, in relation to 69.9 ppm for **2**, **3** and **1**, respectively) and for the (4*S*) model compounds (80.3, 80.6 ppm, in relation to 69.9 ppm for **5**, **6** and **4**, respectively). Glycosylation therefore appears to cause a local electron-withdrawing effect. This is similar in magnitude to the $^{13}C^\gamma$ chemical shifts of ~8 ppm observed on attachment of trifluoroacetate groups to similar model peptides, but more than that due to a simple acyl group, which caused only a shift of ~3 ppm.[33]

NOE experiments: To determine the extent of interaction between the galactose and prolyl rings, selective NOE transfer experiments were performed on the galactosylated model peptides **2**, **3**, **5** and **6** in D_2O (Figure 3). For compound **2**, it was found that selective inversion of Hyp_{β_1} resulted in 0.9 % and 1.2 % resonance transfers to the peaks at $\delta = 3.96$ ppm and $\delta = 3.99$ ppm, which correspond to the galactose H_3 and H_5 protons, respectively. This suggests that α-galactosylation of Hyp results in close contacts between distant positions in the galactose and prolyl rings (Figure 3). The overlap of the hydrophobic α-face of D-galactose with the pyrrolidine ring of proline in **2** resembles other hydrophobic galactose–protein interactions found in several crystal structures.[48,49] In contrast, selective inversion of Hyp_{β_1} in **3** only showed resonance transfer to H_1 of galactose (1.5 %), and no other sugar protons. However, there was greater overlap of the galactose protons in the ^1H NMR spectrum of **3**, which made assignment of NOE contacts with certainty more difficult. Selective inversion of the Hyp_{β_1}/Hyp_{β_2} signal in **5** and **6** only showed an NOE contact to H_1 of galactose (0.7 and 0.4 %, respectively), but not to other sugar protons. Therefore, the galactose rings in the glycosylated (4*S*)-hyp model peptides **5** and **6** are likely to be located distally to the proline rings (Figure 3).

Measurement of $K_{trans/cis}$: The ratios of *trans*/*cis* isomers ($K_{trans/cis}$) were established by integrating as many well-resolved peaks as possible for each isomer, and taking the average for all peaks for each isomer (Table 1).[20] As previously found, glycosylation of (4*R*)-Hyp does not affect $K_{trans/cis}$ in D_2O (**1**–**3** had $K_{trans/cis}$ values of 5.9–6.2).[30] However, it was found that the glycosylation of (4*S*)-hyp does affect $K_{trans/cis}$. The α- and β-linked sugars in **5** and **6**, respectively, were found to stabilize the prolyl *N*-terminal *trans* amide conformation to an equal extent ($K_{trans/cis}$ value of 2.9 in D_2O at 25 °C) relative to **4** ($K_{trans/cis}$ value of 2.4). This is in con-

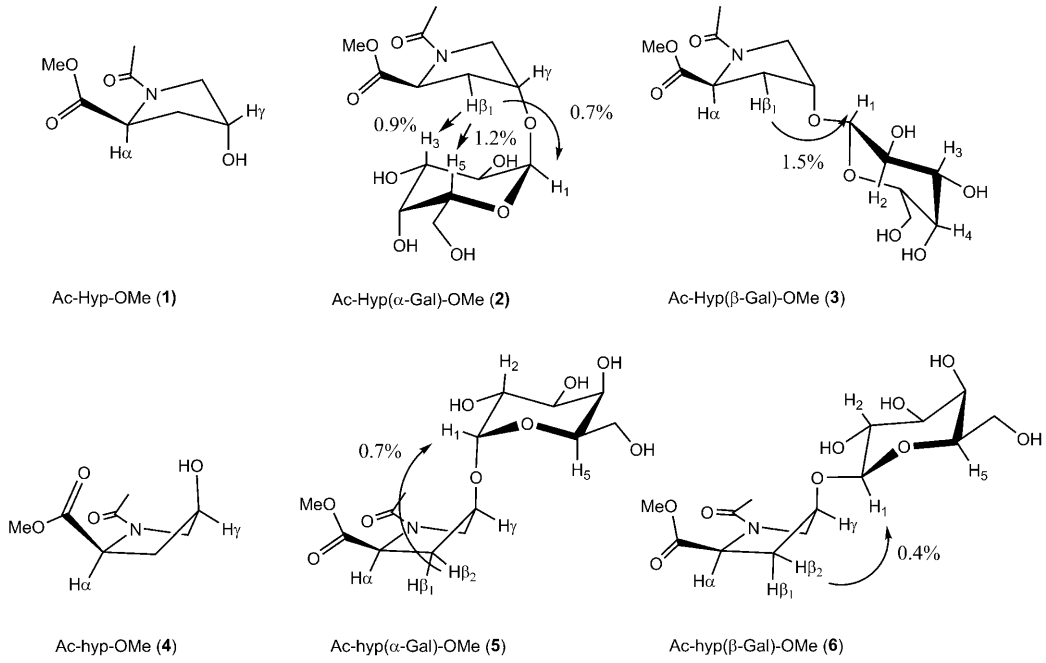


Figure 3. Relevant NOE interactions between the prolyl side chain and the carbohydrate ring for **1**–**6**. These experiments indicate that close contacts between the rings exist when D-galactose is α-linked to Hyp, but not when it is α-linked to hyp. Furthermore, both α- and β-linked D-galactose seem to be oriented away from the proline side chain when *O*-linked to hyp. Thus, Hyp and hyp have different impacts on the orientations of the glycans.

Table 1. Thermodynamic and kinetic parameters for **1**–**6**.

Compound	$K_{trans/cis}$[a]	$\Delta H°_{cis\rightarrow trans}$ [kcal mol^{-1}][b]	$\Delta S°_{cis\rightarrow trans}$ [cal mol^{-1} K^{-1}][b]	$\Delta G°_{300\,K}$ [kcal mol^{-1}][c]	$k_{cis\rightarrow trans}$[d]	$k_{trans\rightarrow cis}$[e]	ν_{amide} [cm^{-1}][f]	$\delta(^{13}C^{\gamma})_{trans}$[g]
1	6.2±0.1	−1.43±0.04	−1.17±0.11	−1.08±0.07	0.81±0.01	0.18±0.01	1612	69.9
2	6.0±0.1	−1.42±0.12	−1.14±0.37	−1.08±0.23	0.85±0.01	0.19±0.01	1611.5	78.9
3	5.9±0.2	−1.45±0.13	−1.38±0.41	−1.04±0.25	0.77±0.02	0.18±0.02	1611.5	77.6
4	2.4±0.1	−0.29±0.08	0.79±0.25	−0.53±0.16	0.44±0.04	0.20±0.01	1612	69.9
5	2.9±0.3	−0.94±0.13	−1.03±0.42	−0.63±0.26	0.59±0.06	0.25±0.03	1611	80.3
6	2.9±0.1	−0.91±0.05	−0.96±0.16	−0.62±0.10	0.71±0.04	0.30±0.02	1613	80.6

[a] Carried out in D_2O at 24.8 °C; ±SE determined by integration of two or more sets of *trans*/*cis* isomers. [b] Error limits obtained by linear least-squares fitting of the van′t Hoff plots to the equation $\ln K_{ct} = (-\Delta H°/R)\ (1/T) + \Delta S°/R$. [c] Calculated from $\Delta G° = \Delta H° - T\Delta S°$. [d] Carried out in phosphate buffer (pH 7.2, 0.1 M) at 67.3 °C; the temperature was calibrated by use of an ethylene glycol standard, and error values were obtained from the linear least-squares fit of the data in Figures S7–S12 in the Supporting Information. [e] Calculated from k_{ct} and amide isomer equilibrium ($K_{t/c}$) at 67.3 °C. [f] Determined in D_2O at 25 °C. [g] Determined by 75 MHz NMR in D_2O at 25 °C.

trast with previous studies of 4-*O*-modification of hyp, which saw a slight stabilization of the *cis* amide isomer. Taylor et al. found that *O*-methylation of hyp in Ac-Phe-(4*S*)-hyp-NHMe model peptides caused a decrease in $K_{trans/cis}$ from 1.9 to 1.4 in D_2O at 25 °C.[36] However, this result is complicated by the prolyl *N*-terminal L-phenylalanine residue, which is known to stabilize the *cis* amide isomer through interaction with the prolyl side chain.[14b] Similarly, Jenkins et al. found that *O*-acylation of hyp in Ac-(4*S*)-hyp-OMe model compounds caused a decrease in $K_{trans/cis}$ from 1.8±0.4 to 1.6±0.2 in D_2O at 25 °C, although these values were within experimental error.[33]

Thermodynamics: The effect of temperature on the $K_{trans/cis}$ values for compounds **1**–**6** was measured by NMR spectroscopy and the resulting van′t Hoff plots are shown in Figure 4. This assumes that the enthalpic and entropic energy differences between the *cis* and *trans* amide isomers are independent of temperature; the linear van′t Hoff plots indicate that this assumption is probably valid.[21,33,38,50] We found in each case that the $K_{trans/cis}$ value decreased with increasing temperature. Compound **4** was slightly anomalous, exhibiting less temperature dependence on $K_{trans/cis}$ than the other model compounds. Accordingly, $\Delta H°$ and $\Delta S°$ could be calculated from the least-squares fits of the van′t Hoff plots. In each case, $\Delta H°$ was <0, which correlates well with other studies of proline model peptides (Table 1).[21,38,51,52] The differences in $K_{trans/cis}$ values were reflected in $\Delta H°$ and $\Delta S°$, with no significant differences in $\Delta H°$ or $\Delta S°$ between compounds **1**, **2** and **3** ($\Delta H°$ values of −1.43±0.04, −1.42±0.12 and −1.45±0.13 kcal mol^{-1}, respectively; $\Delta S°$ values of −1.17±0.11, −1.14±0.37 and −1.38±0.41 cal mol^{-1} K^{-1}, respectively). In contrast, there was an increase of 0.6 kcal mol^{-1} [relative to **4** ($\Delta H°$ of −0.29±0.08 kcal mol^{-1})]

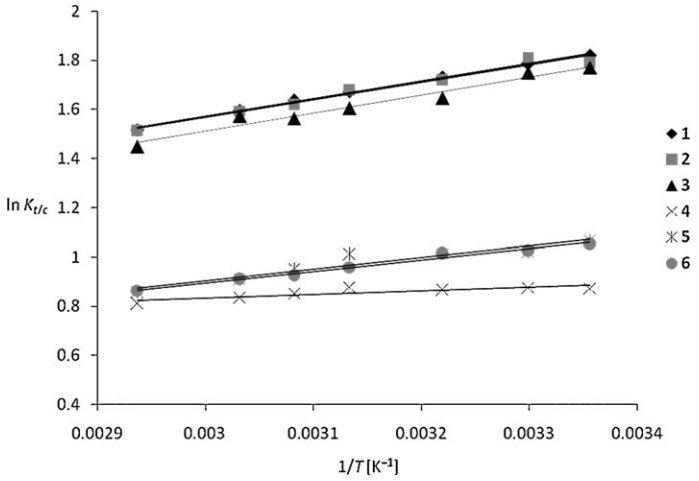


Figure 4. van't Hoff plots for **1–6** in D_2O.

in the enthalpic energy differences in **5** and **6** ($\Delta H°$ values of -0.94 ± 0.13 and -0.91 ± 0.05 kcal mol^{-1}, respectively). Also, there seemed to be different entropic contributions to the ground state energy difference ($\Delta G°$) between the *cis* and *trans* amide isomers, because glycosylation seemed to cause the *trans* amide to become more ordered in **5** and **6** ($\Delta S°$ values of -1.03 ± 0.42 and -0.96 ± 0.16 cal mol^{-1} K^{-1}, respectively), whereas the *trans* amide isomer was more disordered in **4** ($\Delta S°$ of $+0.79\pm0.25$ cal mol^{-1} K^{-1}).

Kinetics: The rates of *cis*/*trans* amide isomerization were calculated for **1–6** with the aid of ^{1}H NMR magnetization inversion transfer experiments in phosphate buffer (pH 7.2, 0.1 M).[21,30,31,53] These experiments were performed at elevated temperature, because at physiological temperature the kinetic rates are too slow to be determined by this assay. It was found that the glycosylated hyp model compounds **5** and **6** exhibited increases in their rates of isomerization ($k_{cis\rightarrow trans}$ values of 0.59 ± 0.06 and 0.71 ± 0.04 s^{-1}, respectively) relative to the unglycosylated model peptide **4** ($k_{cis\rightarrow trans}$ of 0.44 ± 0.04 s^{-1}) at 67 °C (Table 1). This is in contrast with the glycosylated Hyp compounds **2** and **3**, which showed almost no differences in their rates of isomerization ($k_{cis\rightarrow trans}$ values of 0.85 ± 0.02 and 0.77 ± 0.01 s^{-1}, respectively) relative to the unglycosylated model compound **1** ($k_{cis\rightarrow trans}$ of 0.81 ± 0.01 s^{-1}) at 67 °C, which is consistent with previous work.[30]

The changes of ~10 ppm in the $^{13}C^{\gamma}$ chemical shifts of **5** and **6** relative to **4** (and similarly for **2** and **3** relative to **1**) are indicative that glycosylation causes a local electron-withdrawing effect. An increased electron-withdrawing ability has been attributed to stabilization of a given pucker through a stereoelectronic effect.[17,21–25,54] This probably explains why glycosylation does not significantly change the prolyl puckers for **1–3** or **4–6** as determined by the measurement of ^3J coupling constants. However, since changes in $K_{trans/cis}$ have been correlated with changes in prolyl puckers, the stabilization of the *trans* amide isomer in **5** and **6** relative to **4** cannot be explained by such a conformational change in this case.[26,27]

The local electron-withdrawing effect caused by glycosylation may diminish the intramolecular electrostatic repulsion between the 4-hydroxy groups and the *C*-terminal carbonyl oxygen atoms in **5** and **6**.[35] This would allow the prolyl ψ angle to relax from 180° closer to the optimal angle of 150° for a favourable n $\rightarrow$ π^* interaction, which has been estimated to contribute 0.7 kcal mol^{-1} to the stability of the *trans* amide isomer.[22,55] Because this is specific for the C$^{\gamma}$-*endo* conformation, it would explain the lack of effect in **1–3**, which each have a C$^{\gamma}$-*exo* pucker.

The effect of the glycosylation on the intramolecular hydrogen bond in hyp should also be considered, because it has been estimated to contribute 1.5 kcal mol^{-1} to stabilization of the Cγ-*endo* conformation.[35] The presence of the glycosidic linkage would be expected to eliminate this intramolecular interaction, and because NOE experiments indicated that the sugar is not proximal to the prolyl side chain, it is unlikely to be restored through the sugar hydroxy groups. The removal of the intramolecular hydrogen bond should destabilize the Cγ-*endo* conformation in **5** and **6** and thereby destabilize the *cis* amide isomer. This would also explain why there is no impact from glycosylation on the $K_{trans/cis}$ values for **1–3**, because there are no equivalent intramolecular hydrogen bonds. Taylor et al., however, have downplayed the effects of the intramolecular hydrogen bond because *O*-methylation of hyp had little effect on $K_{trans/cis}$ values.[36]

The proposed reductions in the intramolecular electrostatic repulsion and the loss of the intramolecular hydrogen bonds, which have an influence on the enthalpic contributions ($\Delta H°$) to the ground state energy differences ($\Delta G°$) between the *trans* and *cis* amide isomers, seem to be counteracted by decreases in entropy ($\Delta S°$) incurred upon glycosylation in compounds **4–6** (Table 1). In previous work, it has been shown that peptide and protein glycosylation causes more ordered hydration spheres.[56] Here, glycosylation apparently had a further impact on the entropic differences established between the prolyl *trans* and *cis* amide isomers in **4–6**, which have themselves has been attributed to differences in solvation.[52] Interestingly, this effect is less apparent for **1–3**, so the influence of solvation differences seem to be specific to the face of the proline side chain.

NMR magnetization inversion transfer experiments indicated that the hyp model compounds **1–3** have faster amide isomerization rates overall than **4–6**. Improta et al. have calculated that the prolyl nitrogen is more pyramidalized in the Cγ-*exo* pucker than in the Cγ-*endo* pucker,[35] which should facilitate isomerization for **1–3**, which each have a Cγ-*exo* pucker, with respect to **4–6**, each with a Cγ-*endo* pucker. This is in contrast with the findings of Beausoleil et al., who found the reverse effect: hyp had a faster rate than Hyp in Ac-X_{aa}-NHMe model amides in D_2O at 60 °C (2.05 ± 0.50 and 1.46 ± 0.13 s^{-1}, respectively).[31] This was attributed to the intramolecular hydrogen bond in hyp reducing coulombic repulsion between the *C*-terminal carbonyl oxygen atom

and the prolyl nitrogen, although the values were within experimental error.

Glycosylation caused increases in the rates of isomerization of **5** and **6** (0.59 ± 0.04 and $0.71\pm0.06\ s^{-1}$, respectively) relative to **4** ($0.44\pm0.04\ s^{-1}$), whereas no increases in isomerization rates were observed in **1**–**3**. In contrast, the acylation of hyp by Jenkins et al. caused no observable increase in the rate of amide isomerization.[33] Changes in *cis*/*trans* amide isomerization have been correlated to inductive electron-withdrawing effects from prolyl γ-substituents,[21,23] in which the γ-position group withdraws electron density from the peptide bond, thereby increasing *N*-pyramidalization[17,35] and reducing the C–N bond order;[57] this effectively weakens the amide bond and enables isomerization to occur.[36] Whereas glycosylation does appear to cause a local electron-withdrawing effect, it does so both for (4*S*) and for (4*R*) stereoisomers, and does not therefore explain the relative increases in isomerization rates for **5** and **6** relative to **4**, with no changes with **1**–**3**.

An increase in C=O bond order is indicative of a lower C–N bond order, and has been used to explain the increased rate of isomerization of 4-fluoroproline.[23] Here, perhaps because of weaker electron-withdrawing effects, glycosylation either of the (4*R*)- or of the (4*S*)-hydroxyproline model compounds did not affect the amide I vibrational mode maxima, and so also cannot be used to explain the changes in the rates of isomerization of **5** and **6** relative to **4**. Therefore, the basis for the increases in the rates of amide isomerization in **5** and **6** relative to **4**, while in **1**–**3** there is no effect, remains unclear.

Whereas the glycosylation of (4*R*)-hydroxyproline was not found to affect amide isomerization, the glycosylation of (4*S*)-hydroxyproline affects both the prolyl *N*-terminal amide equilibrium and the rate of amide isomerization. Here, we found that both α- and β-anomeric linkages to (4*S*)-hydroxyproline in **5** and **6** stabilize the *trans* amide conformation relative to the unglycosylated model compound **4**. Glycosylation does not significantly alter the Cγ-*endo* conformation of **4** induced by the (4*S*) hydroxylation, but does cause a local electron-withdrawing effect; this probably reduces repulsion between the 4-oxygen atom and the *C*-terminal carbonyl group and allows the ψ angle to relax closer to 150° to restore the $n \rightarrow \pi^*$ interaction that stabilizes the *trans* amide isomer. The changes in $K_{trans/cis}$ for **5** and **6** relative to **4** might also be due to the loss of an intramolecular hydrogen bond that is specific to the (4*S*) stereoisomer. This interaction is probably not restored in **5** and **6** because the sugar was not found to form close contacts to the prolyl side chain. Regardless of their origin, the enthalpic contributions to $\Delta G°$ seem to be offset by entropic changes, which create a more ordered environment for the *trans* amide isomer in **5** and **6** relative to **4**, resulting in only small net changes in $K_{trans/cis}$. The glycosylation of (4*S*)-hydroxyproline does seem to cause a small increase in rate of amide isomerization, but it is not reflected in the amide I vibrational mode and thus the bond order of the *N*-terminal amide group. Therefore, the cause of the increases in the rates remains unclear. Extension of the glycopeptide model amides to larger peptides or to oligohydroxyprolines containing multiple glycosylation sites might result in larger and additive effects, as seen with other oligoproline-based models.[58,59]

The different stereoisomers of 4-hydroxyproline provide an opportunity to understand how each face of the prolyl ring has an influence on $K_{trans/cis}$. Furthermore, Hyp and hyp can be used to project a glycan rigidly in opposite spatial orientations; these building blocks might therefore be useful for studying carbohydrate binding interactions and the influences of glycans on peptide and protein structures in which the orientations of the glycans are important.[60]

Experimental Section

General procedures: Reagent grade solvents were used without further purification. Thin-layer chromatography was performed on precoated silica gel plates (Si250F, 250 μm). Column chromatography was performed on SilicaFlash silica gel (P60, 40–63 μm). NMR spectra were assigned with the aid of 2D COSY and 2D HSQC experiments. For ^{1}H NMR, minor isomers are listed between square brackets. For ^{13}C NMR, when assigned, carbon peaks for the minor isomer are listed in brackets.

General preparation of *N*-acetyl-amino acid methyl esters: The amino acid (1.0 equiv) was dissolved in CH_2Cl_2/piperidine 3:1, stirred for 2 h at ambient temperature and then co-distilled with toluene (3×10 mL). The crude product was then dissolved in acetic anhydride/pyridine 1:1, and the mixture was stirred at ambient temperature for 4 h. The solution was concentrated under reduced pressure and was then co-distilled with toluene (3×10 mL) before purification by flash chromatography.

Preparation of 4-*O*-galactopyranosyl-*N*-acetyl-amino acid methyl esters: The amino acid (1.0 equiv) was dissolved in CH_3OH. The catalyst (20% palladium hydroxide on carbon, approx. 0.5 equiv) was added, and the flask was flushed with N_2 for 5 min. The reaction mixture was then stirred under hydrogen (10 psi) for 6 h, after which it was flushed with nitrogen and filtered. The product was then concentrated under reduced pressure.

***trans*-*N*-Acetyl-4-hydroxy-L-proline methyl ester (1)**: Compound **7** (0.250 g, 1.38 mmol) was dissolved in methanol (2 mL), followed by the addition of triethylamine (0.77 mL, 5.52 mmol, 4.0 equiv) and acetic anhydride (0.78 mL, 8.28 mmol, 6.0 equiv). The reaction mixture was stirred for 2 h at ambient temperature before being concentrated under reduced pressure. The product was purified by flash chromatography with ethyl acetate/methanol 9:1 to yield **1** as a white solid (0.244 g, 1.31 mmol, 95.0%). $[\alpha]_{25}^{D}=-83.3°$ ($c=0.7$, CH_3OH); m.p. 75–78 °C; ^{1}H NMR (500 MHz, D_2O, 298 K): $\delta=$[4.83, dd, $J_{\alpha,\beta_1}=8.0$ Hz, $J_{\alpha,\beta_2}=7.6$ Hz, 0.14 H; Pro_{α}^{cis}], 4.61 (dddd, $J_{\beta_1,\gamma}=2.1$ Hz, $J_{\beta_2,\gamma}=4.4$ Hz, $J_{\gamma,\delta_1}=4.4$ Hz, $J_{\gamma,\delta_2}=1.9$ Hz, 0.86 H; Pro_{γ}^{trans}), 4.54 (dd, $J_{\alpha,\beta_1}=8.4$ Hz, $J_{\alpha,\beta_2}=8.6$ Hz, 0.86 H; Pro_{α}^{trans}), [4.51–4.57, m, 0.14 H; Pro_{γ}^{cis}], 3.85 (dd, $J_{\delta_1,\delta_2}=13.7$ Hz, 0.86 H; $Pro_{\delta_1}^{trans}$), [3.83, s, 0.4 H; $-COCH_3^{cis}$], 3.79 (s, 2.6 H; $-COCH_3^{trans}$), [3.71, dd, $J_{\gamma,\delta_1}=2.2$ Hz, $J_{\delta_1,\delta_2}=12.6$ Hz, 0.14 H; $Pro_{\delta_1}^{cis}$], 3.66 (dd, 0.86 H; $Pro_{\delta_2}^{trans}$), [3.56, dd, $J_{\gamma,\delta_2}=4.5$ Hz, 0.14 H; $Pro_{\delta_2}^{cis}$], [2.50, ddd, $J_{\beta_1,\gamma}=1.7$ Hz, $J_{\beta_1,\beta_2}=13.7$ Hz, 0.14 H; $Pro_{\beta_1}^{cis}$], 2.39 (ddd, $J_{\beta_1,\beta_2}=13.7$ Hz, 0.86 H; $Pro_{\beta_1}^{trans}$), [2.34–2.40, m, 0.14 H; $Pro_{\beta_2}^{cis}$], 2.18 (ddd, 0.86 H; $Pro_{\beta_2}^{trans}$), 2.14 (s, 2.6 H; $-NCOCH_3^{trans}$), [2.03 ppm, s, 0.4 H; $-NCOCH_3^{cis}$]; ^{13}C NMR (75 MHz, D_2O, 298 K): $\delta=$ 172.9, (172.7), (170.8), 170.2, 69.6, (68.0), (58.7), 57.5, 55.9, (54.3), (52.7), 52.2, (39.6), 37.8, 22.1, (21.5 ppm); MS (ES): m/z: calcd for $C_8H_{13}NNaO_4$: 210.07 $[M+Na]^+$; found: 209.73 $[M+Na]^+$.

***trans*-4-*O*-(α-D-Galactopyranosyl)-*N*-acetyl- 4-hydroxy-L-proline methyl ester (2)**: The general preparation method was followed for *O*-debenzylation of **9a** (0.153 g, 0.215 mmol) to yield **2** as a clear oil (0.075 g, 0.215 mmol, quant.). $[\alpha]_{25}^{D}=+105.9°$ ($c=0.3$, CH_3OH); ^{1}H NMR (500 MHz, D_2O, 338 K): $\delta=5.07$ (d, $J=1.9$ Hz, 0.8 H; H_1), [5.03, br d, 0.2 H; H_1], [4.83, dd, $J=7.1$, 7.6 Hz, 0.2 H; Pro_{α}^{cis}], 4.54–4.61 (m, 1.6 H;

Pro_{γ}^{trans}, Pro_{α}^{trans}), [4.51, dddd, J=1.4, 5.0, 5.0, 7.5 Hz, 0.2H; Pro_{γ}^{cis}], 3.98–4.01 (m, 1H; H_5), 3.93–3.98 (m, J=6.0, 6.2 Hz, 1H; H_3), 3.71–3.89 (m, 8.8H; H_2, H_4, H_{6a}, H_{6b}, Pro_{δ_1}, $Pro_{\delta_2}^{trans}$, $-CO_2CH_3$), [3.59, dd, J=5.0, 12.9 Hz, 0.2H; $Pro_{\delta_2}^{cis}$], [2.67, ddd, J=7.5, 7.6, 13.4 Hz, 0.2H; $Pro_{\beta_1}^{cis}$], 2.58 (ddd, J=5.3, 7.9, 13.3 Hz, 0.8H; $Pro_{\beta_1}^{trans}$), [2.43, ddd, J=5.0, 7.1, 13.4 Hz, 0.2H; $Pro_{\beta_2}^{cis}$], 2.23 (ddd, J=5.0, 8.3, 13.3 Hz, 0.8H; $Pro_{\beta_2}^{trans}$), 2.13 (s, 2.4H; $-NCOCH_3^{trans}$), [2.01 ppm, s, 0.6H; $-NCOCH_3^{cis}$]; ^{13}C NMR (75 MHz, D_2O, 298 K): δ=179.6, (179.2), (178.8), 178.2, 102.9, (102.8), 81.3, (79.4), 76.6, (76.5), 74.3, (74.2), 73.0, (72.9), 66.3, (64.1), 62.9, (58.4), 58.3, 58.0, (56.2), 53.9, (41.8), 40.4, 26.3, (25.7 ppm); MS (ES): m/z: calcd for $C_{14}H_{23}NNaO_9$: 372.13 $[M+Na]^+$; found: 372.03 $[M+Na]^+$.

***trans*-4-*O*-(β-D-Galactopyranosyl)-*N*-acetyl-4-hydroxy-L-proline methyl ester (3)**: The general preparation method was followed for *O*-debenzylation of **9b** (0.100 g, 0.141 mmol) to yield **3** as a clear oil (0.049 g, 0.141 mmol, quant.). $[\alpha]_{25}^{D}=-33.6°$ (c=0.5, CH_3OH); ^{1}H NMR (500 MHz, D_2O, 338 K): δ=[4.81, dd, J=7.3, 7.8 Hz, 0.2H; Pro_{α}^{cis}], 4.66–4.73 (m, 0.8H; Pro_{γ}^{trans}), [4.59–4.65, m, 0.2H; Pro_{γ}^{cis}], 4.55 (dd, J=8.2, 8.2 Hz, 0.8H; Pro_{α}^{trans}), 4.49 (d, J=8.0 Hz, 0.8H; H_1^{trans}), [4.47, d, J=8.3 Hz, 0.2H; H_1^{cis}], 3.92–3.99 (m, 1.2H; H_4, $Pro_{\delta_1}^{cis}$), 3.86–3.92 (m, 1.6H; $Pro_{\delta_1}^{trans}$, $Pro_{\delta_2}^{trans}$), [3.85, s, 0.6H; $-CO_2CH_3^{cis}$], 3.74–3.83 (m, 4.4H; H_{6a}, H_{6b}, $-CO_2CH_3^{trans}$), 3.64–3.74 (m, 2H; H_3, H_5), [3.61, dd, J=4.7, 12.9 Hz, 0.2H; $Pro_{\delta_2}^{cis}$], 3.52 (dd, J=8.6, 9.1 Hz, 0.8H; H_2^{trans}), [3.82–3.53, m, 0.2H; H_2^{cis}], [2.60–2.69, m, 0.2H; $Pro_{\beta_1}^{cis}$], 2.55 (ddd, J=2.4, 8.2, 13.4 Hz, 0.8H; $Pro_{\beta_1}^{trans}$), [2.42, ddd, J=5.6, 6.6, 13.4 Hz, 0.2H; $Pro_{\beta_2}^{cis}$], 2.21 (ddd, J=8.2, 10.0, 13.4 Hz, 0.8H; $Pro_{\beta_2}^{trans}$), 2.14 (s, 2.4H; $-NCOCH_3^{trans}$), [2.02 ppm, s, 0.6H; $-NCOCH_3^{cis}$]; ^{13}C NMR (75 MHz, D_2O, 298 K): δ=179.5, (179.1), (178.8), 178.2, (106.8), 106.6, 82.6, (81.3), (80.3), 80.2, (77.65), 77.61, (75.54), 75.48, 73.5, 65.9, (63.7), 62.6, 59.3, (58.4), 58.0, (57.3), (53.9), (41.2), 39.6, 26.3, (25.7 ppm); MS (ES): m/z: calcd for $C_{14}H_{23}NNaO_9$: 372.13 $[M+Na]^+$; found: 372.03 $[M+Na]^+$.

***cis*-*N*-Acetyl-4-hydroxy-L-proline methyl ester (4)**: Compound **10** (0.050 g, 0.204 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (5 mL) and cooled to 0°C, before addition of TFA (5 mL). After stirring for 1.5 h, the solution was co-distilled with toluene (3×6 mL) and concentrated under reduced pressure. The residue was then re-dissolved in methanol (2 mL), followed by the addition of triethylamine (0.112 mL, 0.816 mmol, 4.0 equiv) and acetic anhydride (0.113 mL, 1.224 mmol, 6.0 equiv). The reaction mixture was stirred for 2 h before concentration and purification with ethyl acetate/methanol 9:1 to yield **4** as a clear oil (0.036 g, 0.192 mmol, 94.7%). $[\alpha]_{25}^{D}=+89.9°$ (c=0.7, CH_3OH); ^{1}H NMR (500 MHz, D_2O, 298 K): δ=[4.82, dd, J_{α,β_2}=3.2 Hz, J_{α,β_1}=6.7 Hz, 0.3H; Pro_{α}^{cis}], 4.65 (dd, J_{α,β_2}=2.4 Hz, J_{α,β_1}=9.6 Hz, 0.7H; Pro_{α}^{trans}), 4.57 (dddd, J_{γ,δ_2}=1.0 Hz, $J_{\beta_2,\gamma}$=2.5 Hz, J_{γ,δ_1}=4.6 Hz, $J_{\beta_1,\gamma}$=4.6 Hz, 0.7H; Pro_{γ}^{trans}), [4.51–4.54, m, 0.3H; Pro_{γ}^{cis}], 3.84 (dd, J_{δ_1,δ_2}=11.6 Hz, 0.7H; $Pro_{\delta_1}^{trans}$), [3.80, s, 0.9H; $-CO_2CH_3^{cis}$], 3.77 (s, 2.1H; $-CO_2CH_3^{trans}$), [3.64, dd, J_{γ,δ_1}=4.4 Hz, J_{δ_1,δ_2}=13.4 Hz, 0.3H; $Pro_{\delta_1}^{cis}$], 3.61 (dd, 0.7H; $Pro_{\delta_2}^{trans}$), [3.47, J_{γ,δ_2}=1.0 Hz, 0.3H; $Pro_{\delta_2}^{cis}$], 2.41–2.52 (m, 1.3H; Pro_{β_1}, $Pro_{\beta_2}^{cis}$), 2.22 (ddd, J_{β_1,β_2}=14.2 Hz, 0.7H; $Pro_{\beta_2}^{trans}$), 2.14 (s, 2.1H; $-NCOCH_3^{trans}$), [2.08 ppm, s, 0.9H; $-NCOCH_3^{cis}$]; ^{13}C NMR (75 MHz, D_2O, 298 K): δ=(174.7), 174.5, (174.4), 173.8, 69.9, (68.8), (59.8), 57.9, 56.1, (54.9), (53.5), 53.3, (38.8), 37.3, 21.7, (21.5 ppm); MS (ES): m/z: calcd for $C_8H_{13}NNaO_4$ $[M+Na]^+$: 210.07; found: $[M+Na]^+$: 209.73.

***cis*-4-*O*-(α-D-Galactopyranosyl)-*N*-acetyl-4-hydroxy-L-proline methyl ester (5)**: The general preparation method was followed for *O*-debenzylation of **12a** (0.087 g, 0.123 mmol) to yield **5** as a clear oil (0.043 g, 0.123 mmol, quant.). $[\alpha]_{25}^{D}=+93.6°$ (c=0.3, CH_3OH); ^{1}H NMR (500 MHz, D_2O, 298 K): δ=4.90 (d, J=3.8 Hz, 0.75H; H_1^{trans}), [4.88, d, J=3.8 Hz, 0.25H; H_1^{cis}], [4.69, dd, J=1.1, 9.2 Hz, 0.25H; Pro_{α}^{cis}], 4.53 (d, J=4.3, 7.8 Hz, 0.75H; Pro_{α}^{trans}), 4.34–4.38 (m, 0.75H; Pro_{γ}^{trans}), [4.30–4.33, m, 0.25H; Pro_{γ}^{cis}], 3.78–3.87 (m, 2.5H; H_3^{trans}, H_5, $Pro_{\delta_1}^{trans}$), [3.71–3.75, m, 0.25H; H_3^{cis}], 3.45–3.71 (m, 8.25H; H_2, H_4, H_{6a}, H_{6b}, $Pro_{\delta_1}^{cis}$, Pro_{δ_2}, $-CO_2CH_3$), [2.48–2.55, m, 0.25H; $Pro_{\beta_1}^{cis}$], 2.26–2.37 (m, 1.75H; $Pro_{\beta_1}^{trans}$, Pro_{β_2}), 1.98 (s, 2.25H; $-NCOCH_3^{trans}$), [1.92 ppm, s, 0.75H; $-NCOCH_3^{cis}$]; ^{13}C NMR (75 MHz, D_2O, 298 K): δ=(174.39), (174.34), 174.2, 173.8, 99.1, (98.7), 77.9, (76.3), 72.1, (71.6), 69.75, 69.69, (69.5), 68.4, (68.3), 61.7, (61.3), (59.8), 57.9, 54.5, (53.6), 53.4, (36.4), 35.1, 21.64, (21.59 ppm); MS (ES): m/z: calcd for $C_{14}H_{23}NNaO_9$ $[M+Na]^+$: 372.13; found: $[M+Na]^+$: 372.03.

***cis*-4-*O*-(β-D-Galactopyranosyl)-*N*-acetyl-4-hydroxy-L-proline-methyl ester (6)**: The general preparation method was followed for *O*-debenzylation of **12b** (0.047 g, 0.066 mmol) to yield **6** as a clear oil (0.023 g, 0.066 mmol, quant.). $[\alpha]_{25}^{D}=-40.6°$ (c=0.7, CH_3OH); ^{1}H NMR (500 MHz, D_2O, 338 K): δ=[5.09, dd, J=1.3, 9.2 Hz, 0.3H; Pro_{α}^{cis}], 4.86–4.98 (m, 1.7H; Pro_{γ}, Pro_{α}^{trans}), 4.73 (d, J=8.2 Hz, 0.7H; H_1^{trans}), [4.71, d, J=8.2 Hz, 0.3H; H_1^{cis}], 4.20–4.25 (m, 1H; H_4), 4.16 (dd, J=4.8 Hz. J=11.8 Hz, 0.7H; $Pro_{\delta_1}^{trans}$), 4.02–4.13 (m, 5.7H; H_5, H_{6a}, $Pro_{\delta_2}^{trans}$, $-CO_2CH_3$), 3.90–3.99 (m, 2.6H; H_3, H_{6b}, $Pro_{\delta_1}^{cis}$, $Pro_{\delta_1}^{cis}$), 3.75 (dd, J=7.5, 9.6 Hz, 0.7H; H_2^{trans}), [3.71, dd, J=8.0, 10.0 Hz, 0.3H; H_2^{cis}], [2.90–2.96, m, 0.3H; $Pro_{\beta_1}^{cis}$], 2.79–2.90 (m, 1H; $Pro_{\beta_1}^{trans}$, $Pro_{\beta_2}^{cis}$), 2.67–2.74 (m, 0.7H; $Pro_{\beta_2}^{trans}$), 2.43 (s, 2.1H; $-NCOCH_3^{trans}$), [2.34 ppm, s, 0.9H; $-NCOCH_3^{cis}$]; ^{13}C NMR (75 MHz, D_2O, 298 K): δ=(175.0), 174.7, (174.3), 173.8, 102.3, (102.0), 78.3, (77.0), 75.6, (75.5), 72.9, (71.2), 71.1, 68.9, 61.3, (59.9), 58.0, 54.2, (53.7), 53.5, (52.9), (37.6), 35.9, 21.7, (21.5 ppm); MS (ES): m/z: calcd for $C_{14}H_{23}NNaO_9$: 372.13 $[M+Na]^+$; found: 372.03 $[M+Na]^+$.

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