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The Impact of Pyrrolidine Hydroxylation on the Conformation of Proline-Containing Peptides

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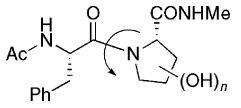

A series of eight dipeptides of the general formula Ac-Phe-Pro*-NHMe was synthesized and the thermodynamics of the cis → trans isomerization about the central amide bond were studied by NMR. Pro* represents the following prolines: L-proline (Pro), L-*trans*-4-hydroxyproline (Hyp), L-*cis*-4-hydroxyproline (hyp), L-*cis*-4-methoxyproline (hyp[OMe]), L-*trans*-3-hydroxyproline (3-Hyp), L-*cis*-3-hydroxyproline (3-hyp), L-2,3-*trans*-3,4-*cis*-3,4-dihydroxyproline (DHP), and L-2,3-*cis*-3,4-*trans*-3,4-dihydroxyproline (dhp). The conformation of the pyrrolidine ring in each case is discussed in light of previous structural studies, analysis of potential stereoelectronic effects, and NMR data. Hydroxy substituents at C-4 have a greater impact on cis → trans isomerization than analogous substituents at C-3 as a result of the intervening bond distances and bridging groups. The position of the equilibrium and its dependence on temperature are a reflection of both enthalpic and entropic factors, the latter being complicated in this study by an Ar-Pro interaction in the cis conformation. The substituents on the pyrrolidine ring determine the conformation of the five-membered ring, which in turn influences the strength of the Ar-Pro interaction, backbone dihedral angles, and the relative energy of the cis and trans species. The ultimate position of the equilibrium depends on a complex blend of steric, electronic, and conformational factors.

1. Background and Introduction

It has long been recognized that proline plays a unique and important role in the conformation of peptides and proteins.[1] Early NMR studies contributed to our understanding the cis−trans isomerism of prolyl amide bonds.[2] Proline residues have been used to good effect in the design of peptides and peptidomimetics with defined conformation.[3]

Nature has produced a plethora of modified prolines,[4] including the hydroxyprolines. When the latter are embedded in a peptide or protein, the hydroxy (OH) substituents on the five-membered pyrrolidine ring provide subtle electronic control, *fine-tuning* the bond angles and orientation of the peptide backbone. Much has been made of the importance of this role in collagen,[5] a structural protein of immense importance that consists of repeating Gly-X-Y triads where X is often Pro (**1**) and Y is often *trans*-4-hydroxyproline (**2**). The importance of hydroxyproline in stabilizing the triple helical structure of collagen was recognized many years ago,[6] but the mechanism by which stabilization is achieved is still evolving and has been the subject of much discussion recently.[5]

It was long-believed that stabilization arose from hydrogen bonds mediated by a network of bridging water molecules, as supported by X-ray crystallographic evidence.[7] More recent structural studies have led to different conclusions.[8] In 1998, Raines proposed that stabilization is achieved principally via the "inductive effect".[9] 4-Fluoroprolines (**4**−**6**)[10] have been widely used

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to probe the effect of electron-withdrawing groups, since fluorine is more electronegative than oxygen, but less capable of hydrogen bonding.[11] Raines and co-workers found that $(Gly\text{-}Pro\text{-}Flp)_{10}$ formed a triple helix that was more stable than $(Gly\text{-}Pro\text{-}Hyp)_{10}$, which in turn was more stable than $(Gly\text{-}Pro\text{-}Pro)_{10}$.[9] This implied that the electron-withdrawing nature of the substituent is the driving force toward stability. *trans*-4-Aminoproline (Amp, **7**) was recently incorporated into a collagenous sequence and shown to afford more stable triple helices than the corresponding peptides containing Hyp (**2**).[12] Babu and Ganesh investigated the amino group because it is both electronegative and has the potential to form hydrogen bonds. However, as they discovered, it has the added complication of being ionizable.

It was observed as early as 1976 that collagen mimetics incorporating *cis*-4-hydroxyproline (hyp, **3**) did not form a triple helix.[13] Thus, the configuration of the stereogenic center bearing the hydroxy substituent is important. This issue has been probed with use of *N*-acetyl amino acid methyl esters (Ac-Pro*-OMe, where Pro* is a substituted proline).[14] Bretscher et al. prepared derivatives incorporating prolines **1** to **5**[14] and Renner et al. prepared derivatives incorporating prolines **4** to **6**.[11d] Both groups concluded that electron-withdrawing 4*R* substituents stabilize the trans conformation of the preceding amide bond. [The terms "cis"and "trans" are used to describe the relative stereochemistry of two substituents on a pyrrolidine ring. The geometry of the peptide bond is also described as being "cis" or "trans". Care should be taken not to confuse these two applications of the same descriptors.] However, the same substituents in the 4*S* orientation shift the equilibrium in the opposite direction. Interestingly, the compound bearing two fluorines at C4 (incorporating **6**) favored the cis conformation slightly more than Pro itself.[11d] As we have recently shown, caution needs to be exercised in utilizing esters of single amino acid residues to draw conclusions about peptide conformation.[15]

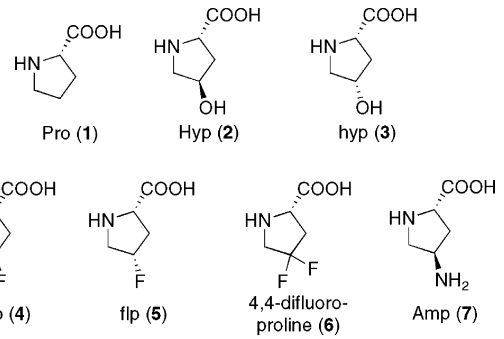

FIGURE 1. Proline and 4-substituted derivatives.

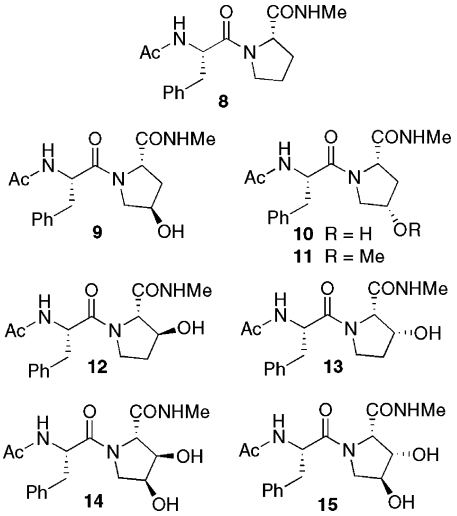

FIGURE 2. Dipeptides synthesized.

Our interest in hydroxylated prolines, and their role in nature, began with our bid to synthesize the repeating decapeptide unit[16] of the mussel adhesive protein, Mefp1,[17] which contains three proline residues in three different oxidation states. As a prelude to studying the conformation of larger peptides containing hydroxylated prolines, we decided to look first at dipeptides, of the general formula Ac-L-Phe-Pro*-NHMe, to consider the empirical effect of pyrrolidine hydroxylation on peptide conformation. This follows from our foundation analysis of the factors contributing to the conformation of X-Pro dipeptides.[15]

2. Results and Discussion

Eight dipeptides of the general formula Ac-Phe-Pro*-NHMe were synthesized (Figure 2). Compound **8**, studied previously by ourselves[15] and others,[18] can be regarded

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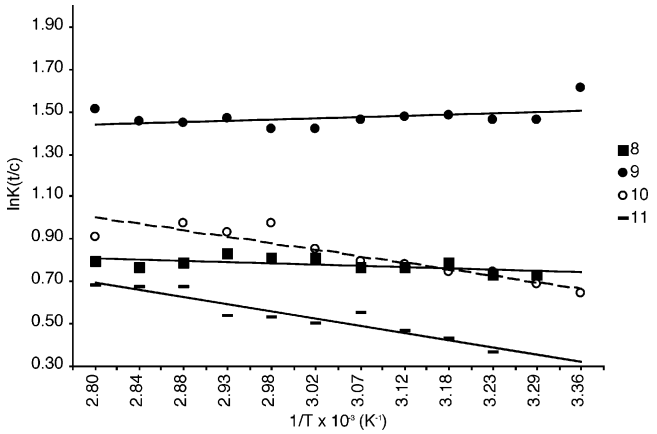

FIGURE 3. Van't Hoff plots for Ac-Phe-Pro-NHMe (**8**), Ac-Phe-Hyp-NHMe (**9**), Ac-Phe-hyp-NHMe (**10**) and Ac-Phe-hyp-(OMe)-NHMe (**11**).

as the parent compound of the series. Compounds **9** and **10** both have a hydroxy group in the 4-position of the proline residue and as such incorporate the much discussed *trans*-4-hydroxyproline residue (Hyp, **2**) and its diastereoisomer, *cis*-4-hydroxyproline (hyp, **3**). We decided to consider the effect of hydroxylation at the 3-position (dipeptides **12** and **13**), since 3-hydroxyprolines have also been isolated from natural sources,[4] including collagens.[19] We prepared dipeptides **14** and **15** incorporating L-2,3-*trans*-3,4-*cis*-3,4-dihydroxyproline (DHP)[17] and L-2,3-*cis*-3,4-*trans*-3,4-dihydroxyproline (dhp),[20] respectively, which were available in our laboratory via synthesis.[21] The dipeptides were synthesized by standard techniques, although the approach varied depending on the availability of each proline derivative and its reactivity. The synthesis and manipulation of the various proline derivatives are included as Supporting Information. Dipeptides were purified by RP-HPLC.

While there are three amide bonds in each molecule, only the central *peptide* bond (between the Phe and Pro residues) was expected to exist as discrete cis−trans conformations on the NMR time scale. As described elsewhere,[15] ^{1}H NMR spectra were assigned on the basis of various 2D experiments, and the equilibrium constants for the cis → trans interconversion in D_2O were determined over a range of temperatures by integration of well-resolved signals in the ^{1}H NMR spectra. The conformation of the pyrrolidine ring influences the cis−trans conformational preference of the X-Pro peptide bond and thereby has an impact on the neighboring peptide backbone.[22] Our results are presented below, along with potential explanations for the differences in behavior.

Let us first consider the introduction of a hydroxy group in the 4-position of the proline residue (Figure 3).

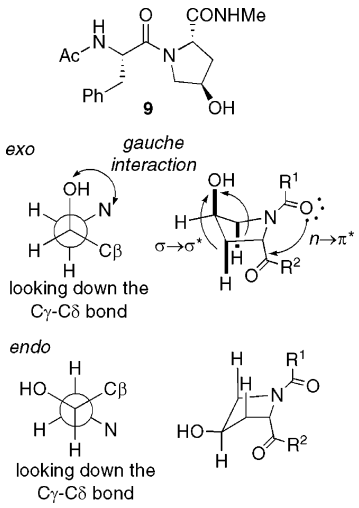

FIGURE 4. Pyrrolidine conformation in dipeptide **9**.

With the introduction of a *trans*-4-hydroxy substituent, there is a significant stabilization of the trans peptide bond: $K_{t/c}$ more than doubles, from $K_{t/c} = 2.1$ in Ac-Phe-Pro-NHMe (**8**) to $K_{t/c} = 5.0$ in Ac-Phe-Hyp-NHMe (**9**) in D_2O at 298 K. Conversely, the introduction of a *cis*-4-hydroxy substituent leads to little change in $K_{t/c}$ values relative to **8**, but there appears to be a slight negative slope of the Van't Hoff plot for Ac-Phe-hyp-NHMe (**10**).

It has long been recognized that the *trans*-4-hydroxy substituent of Hyp is responsible for stabilizing the collagen triple helix[23] and that its diastereomer does not have this capacity. The basis for this is slowly emerging from a number of important new developments.[5] Raines' early work demonstrated that electron-withdrawing groups in the 4-position inductively withdraw electron density from the peptide bond, increasing *N*-pyramidalization, reducing the bond order of the C−N linkage, and thereby facilitating the interconversion of the cis and trans species to favor that which is lower in energy.[11a,b] In the case of *trans*-4-hydroxyproline, the OH group leads to a strong preference for a Cγ-*exo* ("up") conformation of the pyrrolidine ring (Figure 4),[24] and it has been suggested recently that this conformation is desirable in the Y-position of Gly-X-Y triads.[25] This has been attributed by Bretscher et al. to the gauche effect[26]—in the Cγ-*exo* conformation, a gauche orientation of the 4-OH group and the pyrrolidine nitrogen, relative to the Cγ−Cδ bond axis, is possible (Figure 4). Moreover, recent theoretical studies suggest that there is a related hyperconjugative interaction[27] that is the driving force for adopting the Cγ-*exo* conformation: donation from the σ(Cβ−H)$_{ax}$ and σ(Cδ−H)$_{ax}$ bonding orbitals into the σ^*(Cγ−O) antibonding

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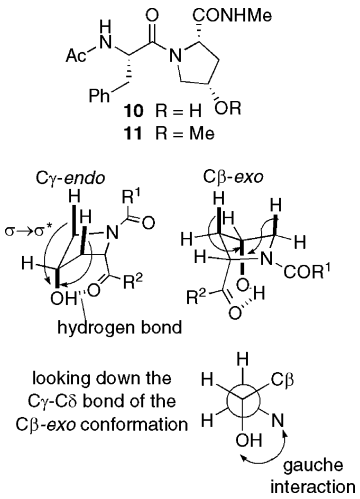

FIGURE 5. Pyrrolidine conformation in dipeptides **10** and **11**.

orbital.[25,27c] Evidence for this stereoelectronic effect is found in the short Cβ−Cγ and Cγ−Cδ bonds (1.512 and 1.510 Å, respectively) and the relatively long C−O bond (1.425 Å) in Ac-Hyp-OMe.[11a] Conversely, a Cγ-*endo* conformation does not allow for such stabilizing effects (Figure 4). A strong preference for the Cγ-*exo* conformation aligns the neighboring peptide backbone[28] for an effective n → π^* electrostatic interaction between the carbonyl groups of the $i - 1$ and i residues when the peptide bond adopts a trans conformation.[14] It has been demonstrated in a recent report by Hinderaker and Raines that this stereoelectronic effect is more important than steric effects which have traditionally been held responsible for the energetic preference for trans peptide bonds.[29]

On the other hand, it has been strongly suggested that introduction of a *cis*-4-hydroxy substituent leads to a preference for the Cγ-*endo* conformation of the pyrrolidine ring (Figure 5).[25] Indeed, Beausoleil et al. have reported structural evidence for a Cγ-*endo* conformation for the pyrrolidine ring of (2*S*,4*S*)-*N*-acetyl-3,3-dimethyl-4-hydroxy-L-proline *N*-methylamide.[30] The *endo* conformation for hyp provides an appealing opposite to the *trans*-4-hydroxy diastereoisomer in terms of stereoelectronic interactions. As illustrated in Figure 5, a gauche effect is achieved in the Cγ-*endo* conformation, along with stabilizing hyperconjugative interactions. However, according to X-ray crystallographic data for "*allo*-4-hydroxy-L-proline dihydrate", it is the Cβ atom that deviates from the plane of the other four atoms of the pyrrolidine ring in the free amino acid, giving rise to a Cβ-*exo* conformation.[31] It was shown early on that pyrrolidine conformations determined by analysis of $^3J_{HH}$ coupling constants from NMR data agree remarkably well with those determined from crystal structures.[32] The dihedral angles in this conformation afford almost the same stabilizing interactions as the Cγ-*endo* conformation (Figure 5).

Interestingly, the Van't Hoff plot for dipeptide **10** demonstrates a slight negative gradient. We speculated that this might be attributable to a hydrogen bond that can form on the lower face of the pyrrolidine ring, between the hydroxy group and the proline amide carbonyl (OH···O=C). This hydrogen bond has been invoked by Bretscher et al. and has been estimated to contribute 1.5 kcal mol^{-1} of stabilization to the *endo* conformation in Ac-hyp-OMe,[14] although Barone and co-workers caution that such intramolecular hydrogen bonds are in competition for intermolecular bonds in polar, protic solvents.[25] To test for the importance of such an intramolecular hydrogen bond in dipeptide **10**, we synthesized dipeptide **15** in which the hydroxyl group is capped as a methyl ether. We need to bear in mind that we are also increasing the size of the substituent, reducing slightly the inductive electron withdrawing nature,[33] and altering the energy of the σ^*(C−O) orbital. Comparison of the Van't Hoff plots for compounds **10** and **11** (Figure 3) indicates lower $K_{t/c}$ values for compound **11**. Such a shift in favor of the cis amide bond might be construed as a stronger preference for the Cγ-*endo* conformation in compound **11**. This downplays the role of the hydrogen bond and the superficial inductive effect in stabilizing the Cγ-*endo* conformation. A recent paper by Alabugin and Zeidan suggests that a σ(C−H)→σ^*(C−OCH$_3$) hyperconjugative interaction is stronger than the corresponding σ(C−H)→σ^*(C−OH) interaction.[27c] They also revealed an inverse correlation in the hyperconjugative acceptor ability of C−X bonds and the electronegativity of X. It would therefore be consistent to propose that there is a stronger σ(C−H)→σ^*(C−OCH$_3$) interaction in compound **11** (relative to the hyperconjugative interaction in dipeptide **10**), which leads to a stronger preference for the Cγ-*endo* conformation and inter alia the cis amide bond.

We next sought to explore the regiochemistry of the pyrrolidine hydroxylation. *trans*-3-Hydroxyproline (3-Hyp) has been indentified in the X-position in collagenous sequences.[19] Recent studies by Jenkins et al. involved the substitution of one modified triad (Gly-*trans*-3-Hyp-*trans*-4-Hyp or Gly-Pro-*trans*-3-Hyp) at the center of 21-mer collagen mimics.[34] Both oligopeptides formed less stable helices than the parent compound containing only Gly-Pro-*trans*-4-Hyp triads, although the effect was less with *trans*-3-Hyp in the X-position.

Jenkins et al. have reported X-ray data for *N*-($^{13}C_2$-acetyl)-3(*S*)-hydroxyproline methyl ester, which showed the conformation of the pyrrolidine ring is such that the Cα, Cγ, and N atoms lie within a plane and Cδ deviates only a little.[34] The carbon bearing the substituent, Cβ, is the "flap" atom of the five membered ring—it points in the opposite direction to the CONHMe group at Cα. In simplified terms, we might refer to this conformation as Cβ-*exo*. Dipeptide **12**, incorporating *trans*-3-hydroxypro-

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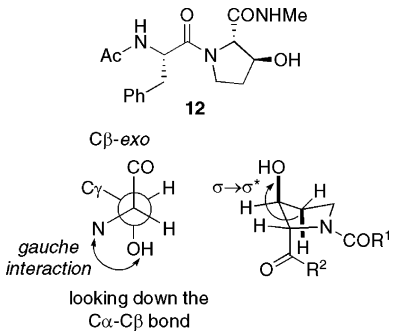


FIGURE 6. Pyrrolidine conformation in dipeptide **12**.

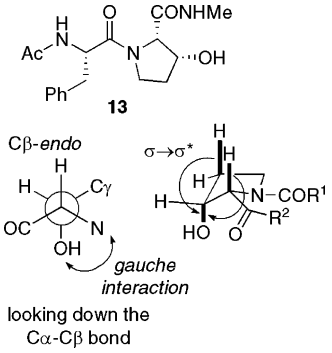


FIGURE 7. Pyrrolidine conformation in dipeptide **13**.

line, is likely to adopt this Cβ-*exo* conformation as a result of a gauche interaction and a stabilizing σ(Cγ−H)→ σ*(Cβ−O) interaction (Figure 6).

For completeness, we also looked at dipeptide **13** containing a *cis*-3-hydroxyproline (3-hyp). This amino acid has not been identified in collagens, but does occur in the cyclic peptide antibiotic telomycin.[35] The *N*-methyl derivative has been isolated in significant quantities from a South Australian *Dendrilla* sponge.[36] Assuming a pyrrolidine conformation in which Cβ is the "flap atom" as discussed above, analysis of the potential stereoelectronic effects predicts that dipeptide **13** would contain a pyrrolidine ring in a Cβ-*endo* conformation (Figure 7).

Van't Hoff plots for dipeptides **12** and **13** alongside the parent compound **8** are given in Figure 8. We are forced to conclude that hydroxylation in the 3-position, regardless of stereochemistry, has a small effect on the conformation of the preceding peptide bond. Why do 3-OH groups have less effect on the cis → trans equilibrium than 4-OH groups? The hydroxy groups are the same number of bonds away from the peptide bond, although the intervening atoms are different. In the case of the 4-OH there is the −CδH$_2$− unit and in the case of the 3-OH there is the −CαH(CONHMe)− bridge. There is structural evidence for a relatively short Cγ−Cδ bond in *N*-acetyl-*trans*-4-hydroxyproline methyl ester;[11a] the shorter distance and the inherent partial double bond character likely account for a strong inductive effect by substituents at C4. On the other hand, the X-ray crystal

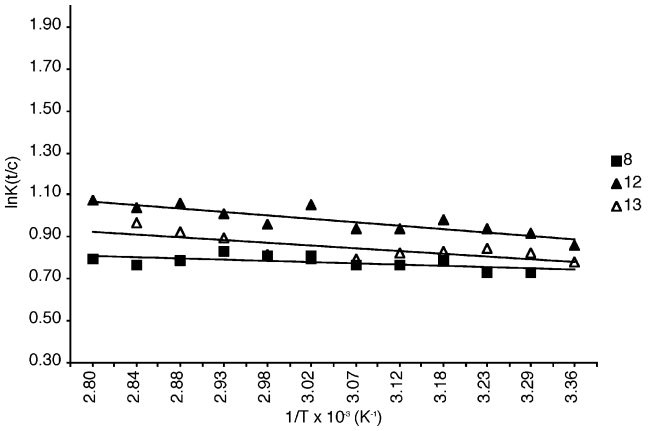


FIGURE 8. Van't Hoff plots for Ac-Phe-Pro-NHMe (**8**), Ac-Phe-*trans*-3-Hyp-NHMe (**12**), and Ac-Phe-*cis*-3-Hyp-NHMe (**13**).

structure of *N*-(^{13}C$_2$-acetyl)-3(*S*)-hydroxyproline methyl ester shows that the Cα−Cβ bond is 1.536 Å, a typical single bond. Consistent with the σ(Cγ−H)$_{ax}$→σ*(Cβ−O) hyperconjugative interaction depicted in Figure 6, the Cβ−Cγ bond length is relatively short (1.512 Å).[34] While both diastereoisomers of the dipeptides containing a 3-hydroxyproline favor the trans rotamer about the Phe-Pro peptide bond relative to the parent compound, the inductive effect is not as strong as in the 4-hydroxyproline-containing compounds. Perhaps the Cβ-*exo* conformation (more than the Cβ-*endo* conformation) of the pyrrolidine ring influences ϕ and ψ dihedral angles in such a way as to strengthen the backbone stereoelectronic interaction in the trans conformation. This would explain the consistently higher $K_{t/c}$ values in dipeptide **13** relative to dipeptide **12**.

To test our developing understanding of the conformation of peptides containing hydroxylated prolines we finally considered dipeptides **14** and **15**, which incorporate 3,4-dihydroxylated prolines.[37] Both have the same relative stereochemistry between C2 and C4, viz. a trans relationship between the CONHMe and 4-OH substituents on the pyrrolidine ring. As such, they share the features of *trans*-4-hydroxyproline which stabilize the Cγ-*exo* pyrrolidine conformation. On the basis of our results for the 3-hydroxyprolines, we might expect the incorporation of a second hydroxy group at C3 to have little influence of the cis → trans equilibrium. This turns out not to be the case, as illustrated by the Van't Hoff plots in Figure 9, and we need to carefully consider how the two hydroxy groups work together to determine the dominant conformation of the pyrrolidine ring and the position of the cis → trans equilibrium.

Van't Hoff plots for dipeptides **14** and **15** are given in Figure 9, along with those of Ac-Phe-Pro-NHMe (**8**) and Ac-Phe-Hyp-NHMe (**9**), which can now both be regarded as "parent compounds." One of the dihydroxyprolines (DHP in dipeptide **14**) displayed similar behavior to *trans*-4-Hyp, giving rise to the largest equilibrium constants in the series ($K_{t/c}$ = 5.6 at 298 K). The diastereoisomer, varying only in configuration at Cβ of the proline

(35) (a) Misiek, M.; Fardig, O. B.; Gourevich, A.; Johnson, D. L.; Hooper, I. R.; Lein, J. *Antibiot. Annu.* **1957**−**1958**, 852−855. (b) Sheehan, J. C.; Mania, D.; Nakamura, S.; Stock, J. A.; Maeda, K. *J. Am. Chem. Soc.* **1968**, *90*, 462−470.

(36) Capon, R. J.; Ovenden, S. P. B.; Dargaville, T. *Aust. J. Chem.* **1998**, *51*, 169−170.

(37) Taylor, S. W.; Waite, J. H. In *Prolyl Hydroxylase, Protein Disulfide Isomerase, and Other Structurally Related Proteins*; Guzman, N. A., Ed.; Marcel Dekker: New York, 1998.

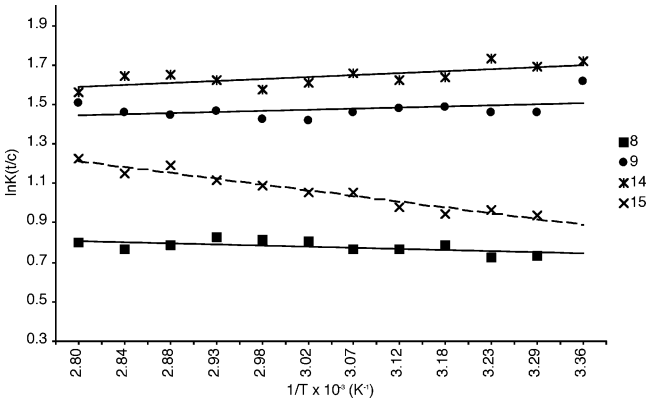
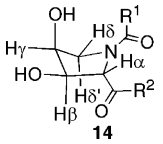


FIGURE 9. Van't Hoff plots for Ac-Phe-Pro-NHMe (**8**), Ac-Phe-Hyp-NHMe (**9**), Ac-Phe-DHP-NHMe (**14**), and Ac-Phe-dhp-NHMe (**15**).

TABLE 1. ^{1}H NMR Data for the Pyrrolidine Ring of Dipeptide 14 in CD_3OD at 298 K and 400 MHz



14

proton	δ (ppm)	multiplicity	coupling constant/s
Hα	4.14	d	$J_{\alpha,\beta} = 5.2$ Hz
Hβ	4.08	dd	$J_{\alpha,\beta} = 5.2$ Hz, $J_{\beta,\gamma} = 4.1$ Hz
Hγ	4.19	app q	$J_{\beta,\gamma} \approx J_{\gamma,\delta} \approx J_{\gamma,\delta'} = 4.5$ Hz
Hδ'	3.62	dd	$J_{\delta,\delta'} = 10.6$ Hz, $J_{\gamma,\delta'} = 5.2$ Hz
Hδ	3.72	dd	$J_{\delta,\delta'} = 10.6$ Hz, $J_{\gamma,\delta} = 4.5$ Hz

residue, led to a significant reduction in $K_{t/c}$ (~2.5 at 298 K) and the Van't Hoff plot displayed a significant temperature dependence.

Dipeptide **14**, incorporating 2,3-*trans*-3,4-*cis*-3,4-dihydroxyproline (DHP), demonstrates similar behavior to dipeptide **9** (containing *trans*-4-hydroxyproline). Indeed, the augmented equilibrium constants are consistent with this dihydroxyproline having a stronger preferance for a Cγ-*exo* conformation than Hyp itself, and therefore a superior alignment of backbone dihedral angles to stabilize the trans peptide bond. No crystallographic data exist for this dihydroxyproline, or derivatives thereof. However, analysis of ^{1}H NMR coupling constants around the pyrrolidine ring in dipeptide **14** supports a Cγ-*exo* conformation. Most significantly, Hγ appears as an apparent quartet, as detailed in Table 1, since it makes approximately the same dihedral angle with Hβ and each of the δ-protons.

Analysis of potential stereoelectronic effects (Figure 10) shows that two additional gauche effects are possible in this conformation of the pyrrolidine ring of **14**, along with the same σ(Cδ−H)$_{ax}$→σ*(Cγ−O) and σ(Cβ−H)$_{ax}$→σ*(Cγ−O) stabilizing interactions as discussed for dipeptide **9**. These additional gauche interaction and the extra inductive electron-withdrawing capabilities of the 3-OH account for the similar but enhanced behavior of **14** relative to **9**.

X-ray crystallographic studies of 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-L-proline (dhp) demonstrated that the pyrrolidine ring exhibited a "Cβ-*endo*" conformation in which Cα, Cγ, Cδ, and N lie in a plane, with a maximum

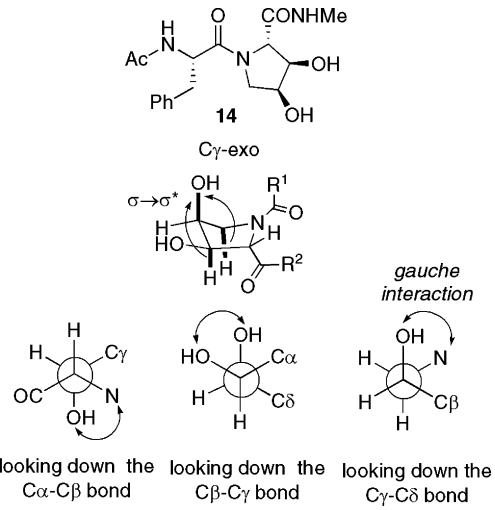


FIGURE 10. Pyrrolidine conformation in dipeptide **14**.

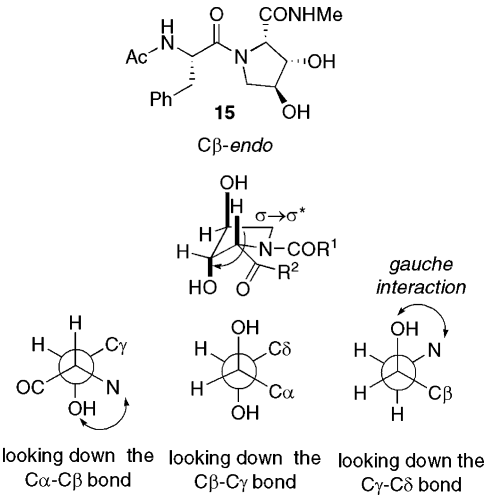


FIGURE 11. Pyrrolidine conformation in dipeptide **15**.

deviation of 0.009 Å. The Cβ atom lies 0.60 Å out of the plane, on the same side of the pyrrolidine ring as the carboxyl group.[38] This means the two hydroxy groups are axial and this conformation can be justified in terms of the kinds of stereoelectronic effects discussed earlier: two gauche interactions are possible and a single σ(Cα-H)$_{ax}$→σ*(Cβ-O) stabilizing interaction (Figure 11). This gives the expectation of a short Cα−Cβ bond, but in fact it is 1.537 Å long in the crystal structure of the free amino acid.[38] The short Cβ−Cγ and Cγ−Cδ bonds (1.518 and 1.517 Å, respectively) are also puzzling.

Thermodynamic parameters for the cis → trans equilibria are presented in Table 2. All compounds demonstrate a small, positive entropy, since the trans species represents a more random conformation. The Ar-Pro interaction and perhaps more extensive hydrogen bonding with solvent by a more exposed amide bond render the cis species more "ordered". The small but positive enthalpies of isomerization for all dipeptides except **9** and **14** indicate that the cis conformation is energetically favored in these cases, presumably by an Ar-Pro interac-

(38) (a) Karle, I. L.; Daly, J. W.; Witkop, B. *Science* **1969**, *164*, 1401−1402. (b) Karle, I. L. *Acta Crystallogr. B* **1970**, *B26*, 765−770.

TABLE 2. Thermodynamic Parameters for Compounds 8−15

compd	$K_{t/c}$[a] D_2O, 298 K	ΔG,[b] 298 K (kcal mol^{-1} K^{-1})	$\Delta\delta$Hα,[c] 298 K	ΔH[d] (kcal mol^{-1})	ΔS[d] (cal mol^{-1} K^{-1})
Ac-Phe-Pro-NHMe (**8**)	2.1	−0.44	−0.73	+0.25	+2.3
Ac-Phe-Hyp-NHMe (**9**)	5.0	−0.90	−0.53	−0.24	+2.2
Ac-Phe-hyp-NHMe (**10**)	1.9	−0.40	−0.15	+1.21	+5.4
Ac-Phe-hyp(OMe)-NHMe (**11**)	1.4	−0.18	−0.81	+1.40	+5.3
Ac-Phe-3Hyp-NHMe (**12**)	2.4	−0.51	−0.44	+0.65	+3.9
Ac-Phe3hyp-NHMe (**13**)	2.2	−0.47	−0.81	+0.48	+3.2
Ac-Phe-DHP-NHMe (**14**)	5.6	−1.01	−0.27	−0.41	+2.0
Ac-Phe-dhp-NHMe (**15**)	2.5	−0.57	−0.63	+0.80	+4.6

[a] Determined by integration of well-resolved signals in the ^{1}H NMR spectrum. [b] Calculated by using $\Delta G = -RT \ln K$. [c] δHα(trans) − δHα(cis). [d] Calculated by using the equation $\Delta G = \Delta H - T\Delta S$ for the line of best fit for the Van't Hoff plot.

tion. Dipeptides **9** and **14** (incorporating *trans*-4-Hyp and 2,3-*trans*-3,4-*cis*-DHP, respectively) exhibit a negative enthalpy; in these two cases the enthalpic and entropic terms work together to produce a significant free energy difference (ΔG) between the cis and trans species.

Wu and Raleigh demonstrated a linear relationship between the equilibrium constant and $\Delta\delta$Hα,[39] the distance by which Hα of the cis conformation is shifted upfield relative to its trans counterpart, as a consequence of the shielding effect of the aromatic ring. While this is undoubtedly valid in the comparison of X-Pro peptide bonds in which a Cγ-*endo* conformation is the norm, there is no direct relationship between $K_{t/c}$ and $\Delta\delta$Hα for the series of compounds discussed herein. Not surprisingly, chemical shift differences are affected by the substitution pattern of the pyrrolidine ring and its conformation.

3. Conclusions

The placement of a secondary alcohol functional group at the β- and γ-positions of a Pro residue has a strong influence of the conformation of the five-membered pyrrolidine ring. The preferred conformation is one in which there are a maximum number of gauche effects and the underlying $\sigma(C-H)_{ax} \rightarrow \sigma^*(C-O)_{ax}$ stabilizing interactions.

The pyrrolidine conformation that has the strongest influence on the position of the cis → trans equilibrium for the preceding X-Pro peptide bond is Cγ-*exo*. This conformation favorably predisposes the peptide backbone to a $n(O_{n-1}) \rightarrow \pi^*((C{=}O)_n)$ Coulombic interaction. The importance of this stereoelectronic effect on peptide conformation is emerging strongly[29] and our results support this. For the first time we have identified another *hydroxylated* proline that stabilizes the trans conformation of the peptide bond better than *trans*-4-Hyp. 2,3-*trans*-3,4-*cis*-3,4-Dihydroxyproline (DHP) has a stronger preference for the Cγ-*exo* conformation and the additional inductive effect of the 3-OH is likely to enhance the pyramidalization of the nitrogen and the electrophilicity of the Pro C=O group.

The results have been difficult to interpret as a result of the Ar-Pro interaction. The importance of this local interaction and its role in protein-folding are also emerging and our results present some new elements to consider. We tentatively suggest that the strength of this interaction depends on how easily the aromatic ring can approach the Cα atom of the Pro residue, and that substituents and pyrrolidine conformation have an influence on this.

In summary, the regiochemistry, stereochemistry and degree of hydroxylation of proline residues has a marked impact on peptide conformation. This work represents the first systematic study in this regard and has produced some surprising results which we have attempted to rationalize in light of other experimental, theoretical, and structural information.

4. Experimental Section

General. See refs 16 and 21 for details. Standard conditions for RP-HPLC purification of dipeptides utilized an Econosil C-18 column (21 mm diameter; 250 mm long) and a flow rate of 12 mL min^{-1}. A gradient method was employed as follows (% acetonitrile in water): 20−30% over 8 min; 30−95% over 2 min; 95% for 5 min; 95−20% over 5 min. The compounds absorbed approximately 10-fold less strongly at 259 nm than they did at 218 nm. NMR assignments are based on the analysis of ^{1}H−^{1}H COSY, HMQC, and HMBC spectra.

Ac-Phe-*trans*-4-Hyp-NHMe (9). I. Fmoc-Phe-*trans*-4-Hyp-NHMe. *N*-Hydroxysuccinimide (30 mg, 0.258 mmol, 1.0 equiv), followed by DCC (53 mg, 0.258 mmol, 1.0 equiv), was added to a solution of Fmoc-Phe-OH (100 mg, 0.258 mmol, 1.0 equiv) in CH_2Cl_2 (3 mL) at 0 °C under N_2. The solution was stirred at 0 °C for 20 min and then warmed to room temperature and stirred for a further 4 h. The suspension was filtered through a plug of cotton in a Pasteur pipet. The filtrate was concentrated to 2 mL and then refrigerated for 2 h. The suspension was filtered again and the residue was evaporated to give a colorless foam that was dissolved in DMF (1.5 mL) and cooled to 0 °C under N_2. L-*trans*-4-Hydroxyproline (34 mg, 0.258 mmol, 1.0 equiv) was added as a solid in one portion, followed by the dropwise addition of diisopropylethylamine (45 μL, 33 mg, 0.258 mmol, 1.0 equiv). The solution was gradually warmed to room temperature and left to stir overnight. The mixture was diluted with ethyl acetate (20 mL) and washed with 2 M HCl (20 mL). The acidic aqueous layer was back-extracted with ethyl acetate (20 mL). The organic layers were combined, washed with water (40 mL) and brine (40 mL), filtered through $MgSO_4$, and concentrated to give Fmoc-Phe-*trans*-4-Hyp-OH. This crude acid was dissolved in CH_2Cl_2 (3 mL) and cooled to 0 °C under N_2. Methylamine hydrochloride (17 mg, 0.258 mg, 1.0 equiv) was added, followed by triethylamine (83 μL, 60 mg, 0.595 mmol, 2.4 equiv) and finally BOP reagent (110 mg, 0.258 mmol, 1.0 equiv). The mixture was stirred at room temperature for 16 h, diluted with CH_2Cl_2 (25 mL), washed with 2 M HCl (30 mL), water (30 mL), and brine (30 mL), filtered through $MgSO_4$, and concentrated. The product was isolated by flash chromatography, eluting with 2−5% MeOH in CH_2Cl_2 to give Fmoc-Phe-*trans*-4-Hyp-NHMe (63 mg, 49%). R_f 0.38 (9:1 CH_2Cl_2−MeOH; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.14−3.31 (m, 1H), 2.69 (d, J = 4.8 Hz, 3H), 2.60−2.67 (m, 1H), 3.69 (d, J = 11.0 Hz, 1H), 4.08−4.42 (m, 5H),

(39) Wu, W.-J.; Raleigh, D. P. *Biopolymers* **1998**, *45*, 381−394.

4.57 (t, J = 7.7 Hz, 1H), 4.69 (dd, J = 14.3, 7.3 Hz, 1H), 5.89 (d, J = 7.9 Hz, 1H), 6.62 (d, J = 4.8 Hz, 1H), 7.11−7.30 (m, 8H), 7.37 (t, J = 7.5 Hz, 2H), 7.60 (t, J = 7.5 Hz, 2H), 7.73 (d, J = 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 67.5 MHz) δ 26.3, 36.4, 38.5, 47.0, 53.7, 55.3, 58.8, 67.0, 69.9, 119.9, 124.9, 125.0, 127.0, 127.6, 129.2, 129.3, 135.5, 141.1, 143.5, 155.8, 171.0, 171.2; HRMS (FAB$^+$, NBA, MeOH) calcd for $(MH)^+$ $C_{30}H_{32}N_3O_5$ 514.2342, obsd 514.2326.

II. Ac-Phe-*trans*-4-Hyp-NHMe (9). Diethylamine (4 mL) was added to a solution of Fmoc-Phe-Hyp-NHMe (266 mg, 0.52 mmol, 1.0 equiv) in acetonitrile (10 mL). The solution was stirred at room temperature for 1 h, concentrated, and then concentrated twice more from acetonitrile. The residue was suspended in dichloromethane (8 mL) and the mixture was cooled to 0 °C. Triethylamine (145 μL, 105 mg, 1.04 mmol, 2.0 equiv) was added followed by acetyl chloride (37 μL, 41 mg, 0.52 mmol, 1.0 equiv). The solution was warmed to room temperature, stirred for 16 h, and concentrated. The residue was filtered through a plug of silica gel washing with ethyl acetate (200 mL), followed by a mixture of 9:1 EtOAc−methanol (300 mL). Finally, 8:2 EtOAc−methanol (200 mL) was passed through the column and the eluent concentrated to give a colorless foam (230 mg) that was further purified by RP-HPLC under the standard conditions (R_T = 4 min) to give Ac-Phe-Hyp-NHMe (39 mg, 23%). ^{1}H NMR (D_2O, 400 MHz, $K_{t/c}$ = 5.0 at 298 K) δ 1.71−1.78 (m, 1H^{cis}, Proβ'^{cis}), 1.87 (s, 3H^{trans}, Ac^{trans}), 1.91 (s, 3H^{cis}, Ac^{cis}), 1.97−2.08 (m, 1H^{trans}/1H^{cis}, Proβ'^{trans}, Proβ^{cis}), 2.11−2.19 (m, 1H^{trans}, Proβ^{trans}), 2.70 (s, 3H^{cis}, NHMe^{cis}), 2.73 (s, 3H^{trans}, NHMe^{trans}), 2.84 (dd, J = 14.0, 9.0 Hz, 1H^{trans}, Pheβ'^{trans}), 2.90 (dd, J = 13.0, 7.2 Hz, 1H^{cis}, Pheβ'^{cis}), 2.98 (dd, J = 13.0, 7.6 Hz, 1H^{cis}, Pheβ^{cis}), 3.12 (dd, J = 14.0, 6.7 Hz, 1H^{trans}, Pheβ^{trans}), 3.36 (dd, J = 12.0, 4.4 Hz, 1H^{cis}, Proδ'^{cis}), 3.56−3.64 (m, 1H^{cis}, Proδ^{cis}), 3.59 (dd, J = 10.8, 6.6 Hz, 1H^{trans}, Proδ'^{trans}), 3.77 (d, J = 10.8 Hz, 1H^{trans}, Proδ^{trans}), 3.93 (dd, J = 8.4, 4.7 Hz, 1H^{cis}, Proγ^{cis}), 4.25 (dt, J = 11.1, 5.5 Hz, 1H^{cis}, Proα^{cis}), 4.41−4.49 (m, 2H^{trans}, Proγ^{trans}, Proα^{trans}), 4.70 (t, J = 7.5 Hz, 1H^{cis}, Pheα^{cis}); 4.84 (dd, J = 9.0, 5.5 Hz, 1H^{trans}, Pheα^{trans}), 7.12−7.32 (m, 5H); ^{13}C NMR (CD_3OD, 100 MHz) δ 22.2 (22.2), 26.4 (26.5), 38.3 (39.8), 38.8 (40.4), 54.2 (53.8), 56.5 (55.0), 60.7 (60.8), 70.9 (68.5), 127.8 (128.1), 129.5 (129.4), 129.5 (129.6), 130.4 (129.8), 138.4 (137.6), 172.7, 172.9, 174.6; HRMS (FAB$^+$, NBA, MeOH) calcd for $(MH)^+$ $C_{17}H_{24}N_3O_4$ 334.1767, obsd 334.1773.

Ac-Phe-*cis*-4-hyp-NHMe (10). I. Fmoc-Phe-*cis*-4-Hyp-(O^{t}Bu)-NHMe. A solution of Fmoc-*cis*-4-Hyp(O^{t}Bu)-NHMe (80 mg, 0.189 mmol, 1.0 equiv) in acetonitrile (1.5 mL) and diethylamine (1.5 mL) was stirred at room temperature under N_2 for 30 min and then concentrated. The residue was concentrated twice more from acetonitrile and then suspended in CH_2Cl_2 (3 mL) under N_2. To this was added Fmoc-Phe-OH (77 mg, 0.199 mmol, 1.15 equiv), diisopropylethylamine (82 μL, 61 mg, 0.473 mmol, 2.5 equiv), and BroP reagent (77 mg, 0.199 mmol, 1.05 equiv). The flask was flushed with N_2, stoppered, and left to stir at room temperature overnight. The mixture was concentrated and the residue applied to a flash column in a minimum volume of CH_2Cl_2. The column was eluted with 3:1 EtOAc−hexanes to afford Fmoc-Phe-*cis*-4-Hyp-(O^{t}Bu)-NHMe (106 mg, 98%). R_f 0.20 (2:1 EtOAc−hexanes); ^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.34 (s, 9H), 1.76−1.90 (m, 1H), 2.07−1.25 (m, 1H), 2.78 (d, J = 4.8 Hz, 3H), 3.26−3.65 (m, 3H), 4.09−4.47 (m, 7H), 6.42 (br d, 1H), 7.29−7.42 (m, 9H), 7.58 (d, J = 7.3 Hz, 2H), 7.76 (d, J = 7.3 Hz, 2H); ^{13}C NMR ($CDCl_3$, 67.5 MHz) δ 26.3, 28.0, 36.8, 39.5 (39.1), 47.1, 53.6 (53.9), 55.6 (55.3), 60.2 (60.0), 67.2 (67.1), 69.5, 74.2, 119.9, 125.0, 125.1, 127.0, 127.3, 127.6, 128.6, 128.7, 129.3, 135.9, 141.1, 143.6, 155.6, 171.3, 171.5; HRMS calcd for $(MH)^+$ $C_{34}H_{40}N_3O_5$ 570.2968, obsd 570.2944.

II. Ac-Phe-*cis*-4-Hyp-NHMe (10). A solution of Fmoc-Phe-*cis*-4-Hyp(O^{t}Bu)-NHMe (106 mg, 0.176 mmol) in acetonitrile (2 mL) and diethylamine (2 mL) was stirred at room temperature under N_2 for 30 min and then concentrated. The residue was concentrated twice more from acetonitrile. The primary amine was isolated by flash chromatography, eluting first with 2:1 EtOAc−hexanes to elute the Fmoc byproducts and then with 9:1 CH_2Cl_2−MeOH to isolate the ninhydrin-active amine (R_f 0.37 in 9:1 CH_2Cl_2−MeOH). The relevant fractions were combined and evaporated down to give a straw-colored oil (58.8 mg). This was dissolved in a mixture of pyridine (1 mL) and acetic anhydride (1 mL) and the berry-red mixture was stirred at room temperature under N_2 overnight. The mixture was concentrated and then the residue dissolved in a mixture of CH_2Cl_2 (1 mL) and TFA (0.5 mL) and stirred for 4 h at room temperature under N_2. The mixture was concentrated and the product isolated by RP-HPLC under the standard conditions (R_T = 9.00 min) to give **10** (38.3 mg; 53% over 3 steps). ^{1}H NMR (400 MHz, D_2O $K_{t/c}$ = 1.9 at 298 K) δ 1.47 (ddd, J = 13.6, 9.1, 4.1 Hz, 1H^{cis}, hypβ'^{cis}), 1.74−1.93 (m, 1H^{trans}/1H^{cis}, hypβ'^{trans}, hypβ^{cis}), 1.81 (s, 3H^{trans}, Ac^{trans}), 1.90 (s, 3H^{cis}, Ac^{cis}), 2.17 (ddd, J = 12.7, 9.3, 4.3 Hz, 1H^{trans}, hypβ^{trans}), 2.55 (s, 3H^{cis}, NHMe^{cis}), 2.58 (s, 3H^{trans}, NHMe^{trans}), 2.83 (dd, J = 12.6, 10.7 Hz, 1H^{cis}, Pheβ'^{cis}), 2.91−2.94 (m, 1H^{cis}, Pheβ^{cis}), 2.93 (d, J = 7.7 Hz, 2H^{trans}, Pheβ'^{trans}, Pheβ^{trans}), 3.19 (d, J = 11.2 Hz, 1H^{trans}, hypδ'^{trans}), 3.28 (d, J = 13.3 Hz, 1H^{cis}, hypδ'^{cis}), 3.42 (dd, J = 13.3, 4.7 Hz, 1H^{cis}, hypδ^{cis}), 3.48 (d, J = 8.9 Hz, 1H^{cis}, hypγ^{cis}), 3.78 (dd, J = 11.2, 4.4 Hz, 1H^{trans}, hypδ^{trans}), 4.14 (t, J = 3.5 Hz, 1H^{cis}, hypα^{cis}), 4.25−4.30 (m, 2H^{trans}, hypγ^{trans}, hypα^{trans}), 4.46 (dd, J = 9.3, 6.1 Hz, 1H^{cis}, Pheα^{cis}), 4.69 (t, J = 6.2 Hz, 1H^{trans}, Pheα^{trans}), 7.10−7.30 (Ar, 5H); ^{13}C NMR (D_2O, 100 MHz) δ 24.2, 28.8, 38.8 and 39.6, (40.8 and 41.2), 55.7 (56.0), 58.3 (57.6), 62.7 (62.3), 72.5 (70.4), 130.2, 130.5, 131.6, 131.9, 132.1, 138.1, 138.7, 175.6, (175.5), 176.3 and 176.6 (175.9 and 176.0); HRMS calcd for $(MH_2)^+$ $C_{17}H_{24}N_3O_4$ 334.1767, obsd 334.1778.

Ac-Phe-hyp(OMe)-NHMe (11). Boc-hyp(OMe)NHMe (17 mg, 0.0625 mmol, 1.0 equiv) was dissolved in a 1:1 (v/v) solution of TFA and dichloromethane (2 mL). The solution was stirred at room temperature for 45 min then concentrated. The residue was left on the pump for 16 h and then dissolved in dry acetonitrile (2 mL). Diphenylphosphoryl azide (18 μL, 23 mg, 0.081 mmol, 1.3 equiv) was added, followed by *N*-acetylphenylalanine (17 mg, 0.081 mmol, 1.3 equiv) and triethylamine (31 μL, 23 mg, 0.220 mmol, 3.5 equiv). The solution was stirred at room temperature under nitrogen for 18 h and then concentrated. The residue was purified by flash column chromatography, eluting 0−20% MeOH in ethyl acetate. The relevant fractions were combined and concentrated and the final product was further purified by RP-HPLC to give Ac-Phe-hyp(OMe)-NHMe (13) (14 mg, 58%). ^{1}H NMR (D_2O, 400 MHz, $K_{t/c} \approx$ 1.4 at 298 K) δ 1.20 (ddd, J = 14.0, 9.2, 4.0 Hz, 1H^{cis}, hypβ'^{cis}), 1.77 (s, 3H^{trans}, Ac^{trans}), 1.82 (s, 3H^{cis}, Ac^{cis}), 1.93 (br d, J = 14.2 Hz, 1H^{trans}, hypβ'^{trans}), 2.03 (d, J = 14.0 Hz, 1H^{cis}, hypβ^{cis}), 2.14 (ddd, J = 14.2, 9.6, 4.7 Hz, 1H^{trans}, hypβ^{trans}), 2.49 (s, 3H^{trans}/3H^{cis}, NHMe^{trans}, NHMe^{cis}), 2.77 (dd, J = 12.5, 10.4 Hz, 1H^{cis}, Pheβ'^{cis}), 2.75−2.84 (m, 2H^{trans}/1H^{cis}, Pheβ'^{trans}, Pheβ^{trans}, Pheβ^{cis}), 2.97 (s, 3H^{cis}, OMe^{cis}), 3.00 (s, 3H^{trans}, OMe^{trans}), 3.29−3.38 (m, 1H^{trans}/2H^{cis}, hypδ'^{trans}, hypδ'^{cis}, hypγ^{cis}), 3.41 (d, J = 8.8 Hz, 1H^{cis}, hypα^{cis}), 3.69−3.76 (m, 1H^{trans}/1H^{cis}, hypδ^{trans}, hypδ^{cis}), 3.93 (br s, 1H^{trans}, hypγ^{trans}), 4.22 (br d, J = 9.8 Hz, 1H^{trans}, hypα^{trans}), 4.38 (dd, J = 10.0, 5.9 Hz, 1H^{cis}, Pheα^{cis}), 4.65−4.71 (m, 1H^{trans}, Pheα^{trans}), 7.04−7.31 (m, 5H, Ar); ^{13}C NMR (D_2O, 100 MHz) δ 24.1 (24.1), 28.8, 36.4 (37.2), 36.4 (37.2), 39.4 (40.7), 55.1 (55.1), 55.7 (56.1), 58.6 (58.1), 62.2 (62.4), 81.7 (79.9), 130.3 (130.5), 131.7 (131.9), 132.0 (132.0), 138.8 (138.1), 176.0, 176.4, 176.5 (175.6, 175.8, 176.0); HRMS (FAB$^+$, glycerol, MeOH) calcd for $(MH)^+$ $C_{18}H_{26}N_3O_4$ 348.192332, obsd 348.191533.

Ac-Phe-*trans*-3-Hyp-NHMe (12). I. Fmoc-Phe-*trans*-3-Hyp-NHMe. *N*-Hydroxysuccinimide (148.5 mg, 1.29 mmol, 1.0 equiv), followed by DCC (266.2 mg, 1.29 mmol, 1.0 equiv), was added to a solution of Fmoc-Phe-OH (500 mg, 1.29 mmol, 1.0 equiv) in CH_2Cl_2 (15 mL) at 0 °C under N_2. The solution was stirred at 0 °C for 20 min and then warmed to room temperature and stirred for a further 4 h. The suspension was filtered through a plug of cotton in a Pasteur pipet. The filtrate was

concentrated to 5 mL and then refrigerated for 2 h. The suspension was filtered again and the residue evaporated to give a colorless foam that was dissolved in DMF (8 mL) and cooled to 0 °C under N_2. L-*trans*-3-Hydroxyproline (169.2 mg, 1.29 mmol, 1.0 equiv) was added as a solid in one portion, followed by the dropwise addition of diisopropylethylamine (225 μL, 166.8 mg, 1.29 mmol, 1.0 equiv). The solution was gradually warmed to room temperature and left to stir overnight. The mixture was diluted with ethyl acetate (100 mL) and washed with 2 M HCl (100 mL). The acidic aqueous layer was back-extracted with ethyl acetate (100 mL). The organic layers were combined, washed with water (200 mL) and brine (200 mL), filtered through $MgSO_4$, and concentrated to give Fmoc-Phe-*trans*-3-Hyp-OH (568 mg, 1.13 mmol; 88%); R_f 0.57 (6:4:1 $CHCl_3$−MeOH−H_2O). This residue was dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C under N_2. Methylamine hydrochloride (77 mg, 1.13 mmol, 1.0 equiv) was added, followed by triethylamine (380 μL, 276 mg, 2.72 mmol, 2.4 equiv) and finally BOP reagent (502 mg, 1.13 mmol, 1.0 equiv). The mixture was stirred at room temperature for 16 h and then diluted with EtOAc (100 mL), washed with 2 M HCl (100 mL), water (100 mL), and brine (100 mL), filtered through $MgSO_4$, and concentrated. The product was isolated by flash chromatography, eluting with 2−5% MeOH in CH_2Cl_2 to give Fmoc-Phe-*trans*-3-Hyp-NHMe (420 mg, 63% over 2 steps). R_f 0.36 (9:1 CH_2Cl_2−MeOH); ^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.70−2.05 (m, 2H), 2.64 (d, J = 7.4 Hz, 3H), 3.01−3.21 (m, 3H), 3.81 (m, 1H), 4.09−4.56 (m, 4H), 4.66−4.85 (m, 1H), 5.96 (d, J = 7.9 Hz, 1H), 6.36 (d, J = 4.8 Hz, 1H), 6.86 (br s, 1H), 7.18−7.30 (m, 7H), 7.37 (t, J = 7.3 Hz, 2H), 7.54 (t, J = 6.4 Hz, 2H), 7.73 (d, J = 7.3 Hz, 2H); ^{13}C NMR ($CDCl_3$, 67.5 MHz) δ 26.2, 32.8, 38.9, 45.5, 47.1, 53.6, 67.0, 68.6, 72.4, 119.8, 125.0, 126.9, 127.1, 127.6, 128.5, 129.3, 135.8, 141.1, 143.5, 14.6, 155.7, 169.5, 171.7; HRMS (FAB$^+$, NBA, CH_2Cl_2) calcd for $(MH)^+$ $C_{30}H_{32}N_3O_5$ 514.2342, obsd 514.2339.

II. Ac-Phe-*trans*-3-Hyp-NHMe (12). Diethylamine (1 mL) was added to a solution of Fmoc-Phe-*trans*-3-Hyp-NHMe (31.4 mg, 0.061 mmol) in acetonitrile (1 mL). The mixture was stirred under N_2 at room temperature, concentrated, and then concentrated twice more from acetonitrile. The residue was dissolved in a mixture of pyridine (1 mL) and acetic anhydride (1 mL) and left to stir at room temperature under N_2 for 48 h. The mixture was evaporated down and then suspended in a mixture of methanol (2 mL) and water (1 mL). Potassium carbonate (15 mg) was added and the mixture was stirred for 2 h, after which time HPLC analysis of the reaction mixture indicated that hydrolysis of the acetate ester was complete. The reaction mixture was freeze-dried to give a beige powder (39.2 mg) that was subjected to RP-HPLC under the standard conditions to isolate the product (R_T = 10.32 min), Ac-Phe-*trans*-3-Hyp-NHMe (14.2 mg; 70% over 3 steps). ^{1}H NMR (400 MHz, D_2O, $K_{t/c}$ = 2.37 at 298 K) δ 1.67 (ddt, J = 14.0, 7.5, 2.6 Hz, 1H^{cis}, Hypγ'^{cis}), 1.79−1.93 (m, 1H^{trans}/1H^{cis}, Hypγ'^{trans}, Hypγ^{cis}), 1.79 (s, 3H^{cis}, Ac^{cis}), 1.81 (s, 3H^{trans}, Ac^{trans}), 2.03 (dddd, J = 17.8, 13.4, 8.4, 4.9 Hz, 1H^{trans}, Hypγ^{trans}), 2.59 (s, 3H^{cis}, NHMe^{cis}), 2.62 (s, 3H^{trans}, NHMe^{trans}), 2.82 (dd, J = 14.0, 8.6 Hz, 1H^{trans}, Pheβ'^{trans}), 2.86 (dd, J = 13.4, 6.9 Hz, 1H^{cis}, Pheβ'^{cis}), 2.90 (dd, J = 13.4, 7.9 Hz, 1H^{cis}, Pheβ^{cis}), 3.03 (dd, J = 14.0, 5.9 Hz, 1H^{trans}, Pheβ^{trans}), 3.40 (dt, J = 10.8, 7.6 Hz, 1H^{cis}, Hypδ'^{cis}), 3.46−3.55 (m, 1H^{trans}/1H^{cis}, Hypδ'^{trans}, Hypδ^{cis}), 3.69 (m, 1H^{cis}, Hypα^{cis}), 3.79 (ddd, J = 8.7, 8.7, 8.2 Hz, 1H^{trans}, Hypδ^{trans}), 4.13 (m, 1H^{trans}, Hypα^{trans}), 4.18−4.30 (m, 1H^{trans}/1H^{cis}, Hypβ^{trans}, Hypβ^{cis}), 4.51 (dd, J = 7.9, 6.9 Hz, 1H^{cis}, Pheα^{cis}), 4.78 (dd, J = 8.6, 6.2 Hz, 1H^{trans}, Pheα^{trans}), 7.13−7.31 (Ar, 5H); ^{13}C NMR (CD_3OD, 67.5 MHz) δ 22.2, 26.5, 34.1, 38.4 (39.0), 46.5 (46.2), 53.8 (54.2), 70.2 (70.8), 74.4 (76.4), 127.7, 129.4, 130.1, 130.3, 138.3, 172.3, 172.6, 172.8; HRMS (FAB$^+$, glycerol) calcd for $(MH)^+$ $C_{17}H_{23}N_3O_4$ 334.177466, obsd 334.177466.

Ac-Phe-*cis*-3-Hyp-NHMe. (13). Boc-3-*cis*-hyp-NHMe (23 mg, 0.094 mmol, 1.0 equiv) was dissolved in a 1:1 (v/v) solution of TFA and dichloromethane (3 mL). The solution was stirred at room temperature for 45 min and concentrated. The residue was dried in vacuo then dissolved in dry acetonitrile (2 mL). Diphenylphosphoryl azide (23 μL, 29 mg, 0.103 mmol, 1.1 equiv) was added, followed by *N*-acetylphenylalanine (22 mg, 0.103 mmol, 1.1 equiv) and triethylamine (40 μL, 29 mg, 0.28 mmol, 3 equiv). The solution was stirred at room temperature under nitrogen for 18 h, then concentrated. The residue was purified by flash column chromatography, eluting with 0−10% MeOH in ethyl acetate. Relevant fractions were combined and concentrated and the final product was isolated from the residue by RP-HPLC to give Ac-Phe-3-*cis*-hypNHMe (13) (8 mg, 26%). ^{1}H NMR (D_2O, 400 MHz, $K_{t/c}$ = 2.19 at 298 K) δ 1.54 (m, 2H^{cis}, hypγ'^{cis}, hypγ^{cis}), 1.74 (s, 3H^{trans}, Ac^{trans}), 1.80 (s, 3H^{cis}, Ac^{cis}), 1.80−1.92 (m, 1H^{trans}, hypγ'^{trans}), 1.94−2.08 (m, 1H^{trans}, hypγ^{trans}), 2.53 (s, 3H^{cis}, NHMe^{cis}), 2.56 (s, 3H^{trans}, NHMe^{trans}), 2.75−2.83 (m, 1H^{trans}/2H^{cis}, Pheβ'^{trans}, Pheβ'^{cis}, Pheβ^{cis}), 3.00 (dd, J = 13.9, 5.5 Hz, 1H^{trans}, Pheβ^{trans}), 3.19−3.31 (m, 1H^{cis}, hypδ'^{cis}), 3.33−3.44 (m, 2H^{cis}, hypδ^{cis}, hypα^{cis}), 3.53 (q, J = 7.6 Hz, 1H^{trans}, hypδ'^{trans}), 3.76 (br q, J = 7.6 Hz, 1H^{trans}, hypδ^{trans}), 3.88 (q, J = 6.4 Hz, 1H^{cis}, hypβ^{cis}), 4.23 (d, J = 6.1 Hz, 1H^{trans}, hypα^{trans}), 4.35−4.50 (m, 1H^{cis}, Pheα^{cis}), 4.43 (q, J = 5.5 Hz, 1H^{trans}, hypβ^{trans}), 4.65−4.71 (m, 1H^{trans}, Pheα^{trans}), 7.04−7.35 (m, 5H′ Ar); ^{13}C NMR (D_2O, 100 MHz) δ 24.2 (24.4), 28.6 (28.6), 35.3 (32.6), 39.2 (41.4), 48.4 (47.3), 55.5 (55.6), 67.9 (67.1), 72.8 (74.1), 130.1 (130.3), 131.6 (131.8), 132.0 (132.0), 139.2 (138.5), 173.0 (173.4), 175.3, 176.6 (175.0, 175.6); HRMS (FAB$^+$, NBA, MeOH) calcd for $C_{17}H_{23}O_4N_3$ $(MH)^+$ 334.1752, obsd. 334.1780.

Ac-Phe-(2,3-*trans*-3,4-*cis*-3,4-dihydroxy)-l-Pro-NHMe (14). I. Fmoc-Phe-(3,4-O-isopropylidine-DHP)-NHMe (DHP = 2,3-*trans*-3,4-*cis*-3,4-dihydroxy-L-proline). Diethylamine (2 mL) was added to a solution of Fmoc-(3,4-di-*O*-isopropylidine)DHP-NHMe (63.9 mg, 0.152 mmol, 1.0 equiv) in acetonitrile (2 mL). The solution was stirred at room temperature under N_2 for 30 min and then concentrated, then concentrated two further times from acetonitrile. The residue was dissolved in CH_2Cl_2 (4 mL) under N_2 and cooled to 0 °C. Fmoc-Phe-OH (64.6 mg, 0.167 mmol, 1.1 equiv) was added, followed by diisopropylethylamine (66 μL, 49 mg, 0.379 mmol, 2.5 equiv) and finally BrOP reagent (64.7 mg, 0.167 mmol, 1.1 equiv). The mixture was gradually allowed to warm to room temperature and stirred for 2 d. The mixture was concentrated and the residue applied to a flash column in a minimum volume of CH_2Cl_2. The column was eluted with 3:1 EtOAc−hex to isolate Fmoc-Phe-(3,4-*O*-isopropylidine)DHP-NHMe (65.2 mg; 75%). R_f 0.25 (2:1 EtOAc−hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.26 (s, 3H), 1.35 (s, 3H), 2.76 (s, 3H), 2.93 (dd, J = 12.3, 4.8 Hz, 1H), 3.04 (d, J = 6.7 Hz, 1H), 3.83 (d, J = 12.4 Hz, 1H), 4.21 (t, J = 7.1 Hz, 1H), 4.34 (dd, J = 10.9, 3.6 Hz, 1H), 4.46 (dd, J = 10.6, 7.1 Hz, 1H), 4.73 (t, J = 5.3 Hz, 1H), 4.80 (dd, J = 14.9, 6.6 Hz, 1H), 5.04 (d, J = 6.0 Hz, 1H), 5.67 (d, J = 8.2 Hz, 1H), 6.29 (d, J = 4.6 Hz, 1H), 7.09 (dd, J = 7.7, 1.8 Hz, 2H), 7.18−7.43 (m, 7H), 7.58 (d, J = 7.5 Hz, 2H), 7.77 (d, J = 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.7, 26.1, 26.6, 38.9, 47.1, 52.7, 53.1, 65.4, 67.0, 79.3, 80.0, 111.9, 120.0, 125.0, 125.1, 127.0, 127.2, 127.7, 128.6, 129.4, 135.5, 141.3, 143.7, 143.8, 155.3, 168.5, 170.7; HRMS (FAB$^+$, glycerol, MeOH) calcd for $(MH)^+$ $C_{33}H_{34}N_3O_6$ 570.2604, obsd 570.2613.

II. Ac-Phe-DHP-NHMe (14). Diethylamine (1.5 mL) was added to a solution of Fmoc-Phe-(3,4-*O*-isopropylidine)DHP-NHMe (34.7 mg, 0.061 mmol) in acetonitrile (1.5 mL). The mixture was stirred under N_2 for 30 min, concentrated, and then concentrated twice more from acetonitrile. The residue was applied to a flash column in a minimum volume of CH_2Cl_2 and then eluted with 2:1 EtOAc−hexanes, to elute the Fmoc-piperidine adduct, then the polarity increased to 9:1 CH_2Cl_2−MeOH to elute the primary amine (R_f 0.41, 9:1 CH_2Cl_2−MeOH) (17.4 mg). The amine was dissolved in pyridine (1 mL) and acetic anhydride (1 mL) and stirred at room temperature under N_2 overnight. The mixture was concentrated then dissolved in CH_2Cl_2 (2 mL) and TFA (1 mL). The mixture was stirred under N_2 at room temperature for 4 h and then

concentrated. The brown residue was subjected to RP-HPLC according to the standard conditions (R_T = 7.52 min) to isolate Ac-Phe-DHP-NHMe (12.9 mg, 61% over 3 steps). ^{1}H NMR (400 MHz, D_2O, $K_{t/c}$ = 5.58 at 298 K) δ 1.80 (s, 3H^{trans}/3H^{cis}, Ac^{cis}, Ac^{trans}), 2.63 (s, 3H^{cis}, NHMe^{cis}), 2.76 (s, 3H^{trans}, NHMe^{trans}), 2.80 (dd, J = 13.5, 7.2 Hz, 1H^{cis}, Pheβ'^{cis}), 2.79 (dd, J = 13.9, 8.6 Hz, 1H^{trans}, Pheβ'^{trans}), 2.89 (dd, J = 13.5, 7.6, 1H^{cis}, Pheβ^{cis}), 2.99 (dd, J = 13.9, 5.9 Hz, 1H^{trans}, Pheβ^{trans}), 3.34 (dd, J = 12.5, 4.5 Hz, 1H^{cis}, DHPδ'^{cis}), 3.52−3.58 (m, 1H^{cis}, DHPδ^{cis}), 3.54 (dd, J = 11.6, 4.4 Hz, 1H^{trans}, DHPδ'^{trans}), 3.72 (dd, J = 11.5, 2.9 Hz, 1H^{trans}, DHPδ^{trans}), 3.74 (d, J = 4.9 Hz, 1H^{cis}, DHPα^{cis}), 4.01 (d, J = 6.7 Hz, 1H^{trans}, DHPα^{trans}), 4.05−4.15 (m, 1H^{trans}/2H^{cis}, DHPγ^{trans}, DHPβ^{cis}, DHPγ^{cis}), 4.19 (ddd, J = 7.1, 6.7, 3.5 Hz, 1H^{trans}, DHPβ^{trans}), 4.50 (dd, J = 7.6, 7.2 Hz, 1H^{cis}, Pheα^{cis}), 4.73 (dd, J = 8.6, 5.9 Hz, 1H^{trans}, Pheα^{trans}), 7.09−7.33 (Ar, 5H); ^{13}C NMR (D_2O, 100 MHz) δ 24.2 (24.3), 28.8 (28.9), 39.3 (41.0), 54.9, 55.1, (53.6, 53.9), 67.6 (68.5), 73.1 (71.1), 76.8 (78.4), 130.0, 131.5, 131.7, 131.9, 132.2, 139.0, 138.7), 174.8, 175.2, 176.5.

Ac-Phe-(2,3-*cis*-3,4-*trans*-3,4-dihydroxy-l-Pro)-NHMe (15). I. Fmoc-L-Phe-dhp(OTBDMS)$_2$-NHMe (dhp = 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-L-proline). Fmoc-dhp(OTBDMS)$_2$-NHMe (41.8 mg, 0.068 mmol, 1.0 equiv) was stirred in a mixture of acetonitrile (1 mL) and diethylamine (1 mL) for 30 min at room temperature under N_2. The mixture was concentrated, then concentrated from acetonitrile two more times. The residue was suspended in CH_2Cl_2 (2.5 mL). To this mixture was added Fmoc-Phe-OH (29.2 mg, 0.075 mmol, 1.1 equiv), diisopropylethylamine (30 μL, 22 mg. 0.171 mmol, 2.5 equiv), and BrOP reagent (29.2 mg, 0.075 mmol, 1.1 equiv). The mixture was stirred at room temperature under N_2 for 20 h and then concentrated. The residue was applied to a flash column in a minimum volume of CH_2Cl_2 and then eluted with 2:1 hexanes−EtOAc, increasing the polarity to 1:1 hexanes−EtOAc to isolate Fmoc-Phe-dhp(OTBS)$_2$-NHMe (48.8 mg, 94%). R_f 0.39 (1:1 hexanes−EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz, major rotamer) δ −0.02−0.09 (m, 12H), 0.78 (s, 15H), 0.86 (s, 3H), 2.57 (d, J = 4.9 Hz, 3H), 3.11 (dd, J = 13.5, 9.0 Hz, 1H), 3.17 (dd, J = 13.5, 5.3 Hz, 1H), 3.41 (dd, J = 10.2, 3.0 Hz, 1H), 3.90 (br s, 1H), 4.14−4.31 (m, 3H), 4.33−4.43 (m, 2H), 4.72 (app q, J = 4.8 Hz, 1H), 4.91 (td, J = 8.5, 5.3 Hz, 1H), 5.75 (d, J = 9.4 Hz, 1H), 7.18−7.42 (m, 9H), 5.57−7.60 (m, 2H), 7.73−7.78 (m, 2H); ^{13}C NMR ($CDCl_3$, 67.5 MHz) δ −5.1, −4.9, −4.6, 17.7, 17.8, 25.5, 25.6, 26.1, 39.5, 47.1, 53.4, 54.4, 65.7, 67.2, 74.8, 76.4, 119.9, 125.0, 127.0, 127.1, 127.3, 127.5, 127.6, 128.6, 129.0, 129.1, 136.2, 141.1, 143.6, 143.7, 155.3, 167.4, 171.7; HRMS calcd for M$^+$ ($C_{42}H_{59}N_3O_6Si_2$) 758.4021, obsd 758.4045.

II. Ac-L-Phe-2,3-*cis*-3,4-trans-3,4-dihydroxy-L-proline methyl amide (15). A solution of Fmoc-Phe-dhp(OTBS)$_2$-NHMe (4.8 mg, 0.006 mmol) in acetonitrile (0.8 mL) and diethylamine (0.8 mL) was stirred at room temperature under N_2 for 30 min and then concentrated. The residue was concentrated twice more from acetonitrile. The residue was suspended in pyridine (0.5 mL) and acetic anhydride (0.5 mL) and left to stir under an atmosphere of N_2 overnight. The reaction mixture was concentrated and the residue dissolved in THF (2 mL) and a solution of TBAF (20 μL, 1 M in THF) was added. After 100 min, the mixture was concentrated and the product isolated by RP-HPLC under standard conditions (R_T = 9.0 min) to give compound **15** (2 mg, 91% over 3 steps). ^{1}H NMR (400 MHz, D_2O, $K_{t/c}$ = 2.81 at 298 K) δ 1.80 (s, 3H^{trans}, Ac^{trans}), 1.85 (s, 3H^{cis}, Ac^{cis}), 2.59 (s, 3H^{cis}, NHMe^{cis}), 2.62 (s, 3H^{trans}, NHMe^{trans}), 2.78−2.86 (m, 1H^{cis}, Pheβ'^{cis}), 2.82 (dd, J = 14.1, 9.0 Hz, 1H^{trans}, Pheβ'^{trans}), 2.98−3.08 (m, 1H^{cis}, Pheβ^{cis}), 3.03 (dd, J = 14.1, 5.9 Hz, 1H^{trans}, Pheβ^{trans}), 3.19 (dd, J = 12.6, 4.4 Hz, 1H^{cis}, dhpδ'^{cis}), 3.60 (dd, J = 12.6, 5.7 Hz, 1H^{cis}, dhpδ^{cis}), 3.63−3.78 (m, 2H^{trans}/2H^{cis}, dhpδ'^{trans}, dhpδ^{trans}, dhpα^{cis}, dhpγ^{cis}), 3.99 (dd, J = 9.9, 4.7 Hz, 1H^{cis}, dhpβ^{cis}), 4.14 (dd, J = 7.9, 4.0 Hz, 1H^{trans}, dhpγ^{trans}), 4.21 (dd, J = 6.2, 4.0 Hz, 1H^{trans}, dhpβ^{trans}), 4.39 (d, J = 6.2 Hz, 1H^{trans}, dhpα^{trans}), 4.48 (dd, J = 8.5, 6.5 Hz, 1H^{cis}, Pheα^{cis}), 4.75 (dd, J = 9.0, 5.9 Hz, 1H^{trans}, Pheα^{trans}), 7.09−7.31 (Ar, 5H); ^{13}C NMR (D_2O, 100 MHz) δ 24.2 (22.8), 28.5, 39.3 (41.3), 54.7 (53.5), 55.6 (55.3), 66.5 (71.3), 76.6 (74.5), 77.0 (78.3), 130.0, 130.2, 131.5, 131.8, 131.9, 132.0, 138.4, 139.1, 172.8, 175.5, 176.5.

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Supporting Information Available: Tables of thermodynamic data and selected NMR spectra for compounds **9**−**15** and intermediates in their synthesis; procedures and characterization data for the preparation of the following proline building blocks: *N*α-Fmoc-*cis*-4-*tert*-butyloxy-L-proline methyl amide, *N*α-Boc-*cis*-4-methoxy-L-proline methyl amide, (2*S*,3*R*,4*S*)-*N*α-Fmoc-3,4-isopropylidine-3,4-dihydroxy-L-proline methyl amide, and (2*S*,3*S*,4*S*)-*N*α-Fmoc-3,4-*tert*-butyldimethylsilyloxy-L-proline methyl amide. This material is available free of charge via the Internet at http://pubs.acs.org.

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