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Conformational Studies and Stereochemical Assignment of a Bicyclic Lactam-Containing Peptide Fragment by Two-Dimensional NMR Spectroscopy

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The stereochemistry and conformation of a key bicyclic lactam-based Leu–Pro building block and the conformation of the surrounding peptide fragment were assigned using a combination of 2D-NOE data and coupling constants from an NMR simulation. The work confirmed that the initial stereochemistry of the building block had not been lost during its incorporation into the peptide. The proline portion of the building block was found to be in a predominantly *endo* conformation. The six-membered ring lactam that was used to constrain the leucine portion of the building block was found to be in a half-chair conformation. The peptide fragment on both sides of the building block was found to be in an extended conformation.

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INTRODUCTION

Recent developments in molecular modeling have led to a host of predictions concerning the biologically active conformation of peptides and peptide-based drug candidates. But how do we know that these predictions are accurate? This question has led a number of groups to begin examining the use of conformationally restricted peptide analogs as potential chemical probes for determining the relationship between the predicted and actual biological activity of peptide conformations.[1] For our part, these efforts have been focused on the use of bicyclic lactam rings as conformational constraints.[2–4] An example of this approach is illustrated in Scheme 1.[2f] In this example, a *trans* peptide bond mimetic was constructed by imbedding the peptide backbone into the bicyclic ring system. In principle, the conformation of the peptide backbone can be adjusted by altering the size of the two rings used as constraints and by controlling the relative stereochemistry of the substituents on the ring and the bridgehead carbon.

For peptide fragments such as **2** to function as effective conformational probes, the stereochemistry of the

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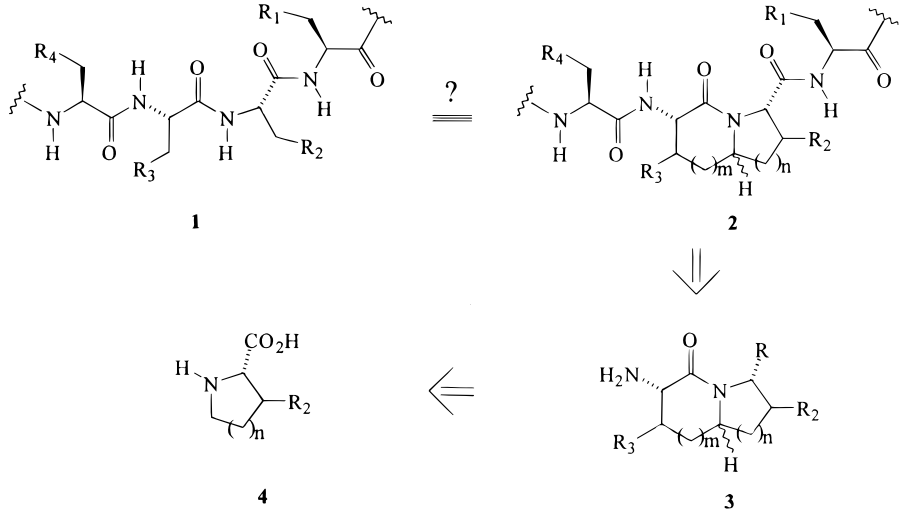


Scheme 1

CCC 0749–1581/97/040267–06 $17.50

stereogenic atoms must be unequivocally assigned. This is especially true for the stereochemistry about the five-membered ring, where a change in stereochemistry has been shown to afford dramatic changes in biological activity.[4b] To date these efforts have focused on assigning the stereochemistry of the building blocks.[2f,5] It has been assumed that the stereochemistry of the building block was preserved during its incorporation into the peptide fragment. The stereochemistries of **5a** and **5b** (Scheme 2) were assigned using 2D-NOE experiments. After conversion to **6a** and **6b**, were all of the stereocenters still the same?

The center on the proline ring went through several points where it could have epimerized. If the stereochemistry were not the same, then any conclusions about a receptor-bound conformation based on the mimetics would be in jeopardy. In addition, we hoped to gain insight into how the incorporation of the bicyclic lactam ring skeleton into the peptide fragment influenced the conformation of the peptide chain surrounding the conformational constraint. The increased complexity of the NMR spectrum for **6a**,**b** relative to **5a**,**b** made the approach used for the assignment of **5a**,**b** difficult. We report here that the stereochemistry and peptide conformation of **6b** can be readily assigned using a combination of 2D-NOE data and coupling constants from an NMR simulation. The stereochemistry of **5b** was not altered during incorporation of the building block into the peptide chain.

EXPERIMENTAL

NMR spectra were recorded with a Varian (Palo Alto, CA, USA) Unity-600 spectrometer and the data were processed off-line on a SPARC 10 station with VNMR software. Proton and carbon chemical shifts were measured in ppm downfield from an internal TMS standard. Proton spectra were obtained in a 5200 Hz spectral width collected into 64 K data points with 5.0 s preacquisition delay. Carbon spectra were obtained with a 32 000 Hz spectral width collected into 64 K data points.

The total correlation (2D HOHAHA) spectra[6] were recorded using an MELV-17 mixing sequence of 100 ms, flanked by two 2 ms trim pulses, using the hypercomplex method. A total of $2 \times 320 \times 2048$ data matrix with 16 scans per t_1 value were collected. Gaussian line broadening and a sine-bell function were used in weighting the t_2 and t_1 dimensions, respectively. After two-dimensional Fourier transformation, the spectra resulted in 2048×2048 data points, which were then phase and baseline corrected in both dimensions.

A two-dimensional COSY[7] spectrum was collected into a 512×2048 data matrix with 16 scans per t_1 value. The time domain data were zero filled to yield a 2048×2048 data matrix and Fourier transformed using a sine-bell weighting function in both the t_2 and t_1 dimensions.

The NOESY[8] spectrum resulted from a $2 \times 512 \times 2049$ data matrix with 32 scans per t_1 value. Spectra were recorded with 250 and 500 ms mixing times. The use of a 500 ms mixing time (at 298 K) afforded a better result. The time domain data were zero filled to yield a 2 K × 2 K data matrix and were processed in a similar way to the 2D HOHAHA spectrum described above.

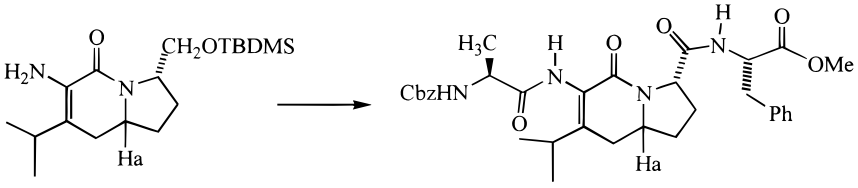
A hypercomplex proton-detected heteronuclear multiple quantum coherence (HMQC)[9] spectrum was recorded using a 0.35 s in BIRD nulling period. The 90° ^{1}H pulse width was 12.5 μs and the 90° ^{13}C pulse width was 22 μs. The proton spectral width was set to 5200 Hz and the carbon spectral width was set to 12 700 Hz. A $2 \times 256 \times 2048$ data matrix with 64 scans per t_1 value was collected. Gaussian line broadening was used in weighting both the t_2 and the t_1 dimensions. After two-dimensional Fourier transformation, the spectra resulted in 512×2048 data points, which were phase and baseline corrected in both dimensions.

RESULTS AND DISCUSSION

'Proton connectivity' assignments

The proton chemical shifts for **6b** were assigned by analysis of COSY, HMQC, HOHAHA and NOESY spectra (Figs 1 and 2). The nomenclature used for discussing this assignment is illustrated in Scheme 3.

The Ala and Phe residues were first identified by the spin propagation from the NH protons through the β-protons. The correlated proton resonances at 4.5, 3.6 and 2.4–1.6 ppm were then assigned to the protons on the bicyclic lactam ring skeleton. The sequential assignment of the amino acid sequence in **6b** was obtained by using the NH_i–αH_{i-1} correlations of the NOESY spectrum (Fig. 2). The observed $d(NH_7$–$H_4)$ confirmed the Ala–six-membered ring connection while the observed $d(NH_{16}$–$H_\alpha)$ confirmed the Pro–Phe connection. A continuous spin propagation from H_ε to H_α indicated that **6b** had the suggested fused 5,6-bicyclic lactam ring skeleton. Using these data it was possible to assign all of the resonances in the proton and carbon NMR spectra to specific residues in **6b**.



5a. Ha=α
5b. Ha=β

6a,b

Scheme 2

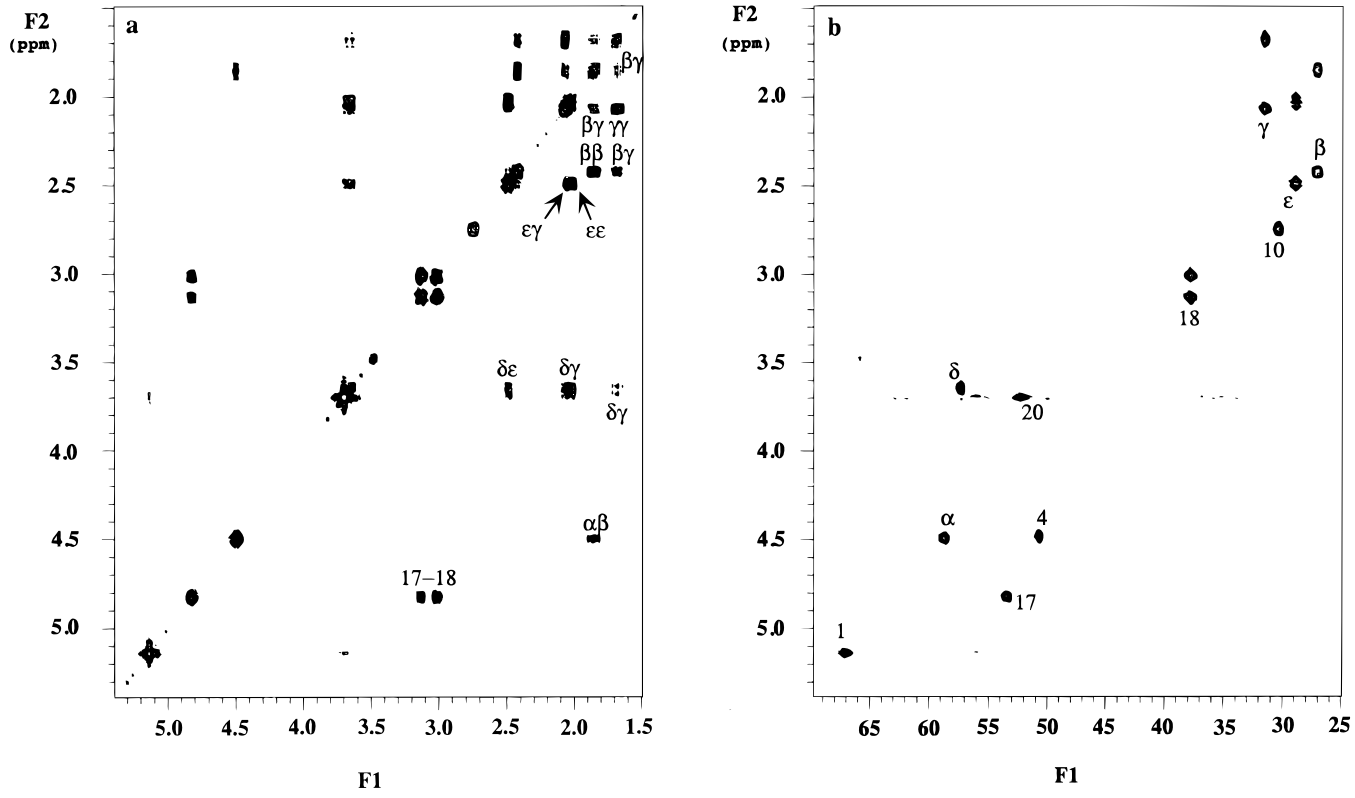


Figure 1. Expansions of the fused ring region of the 600 MHz (a) COSY and (b) HMQC spectra of **6b** in $CDCl_3$.

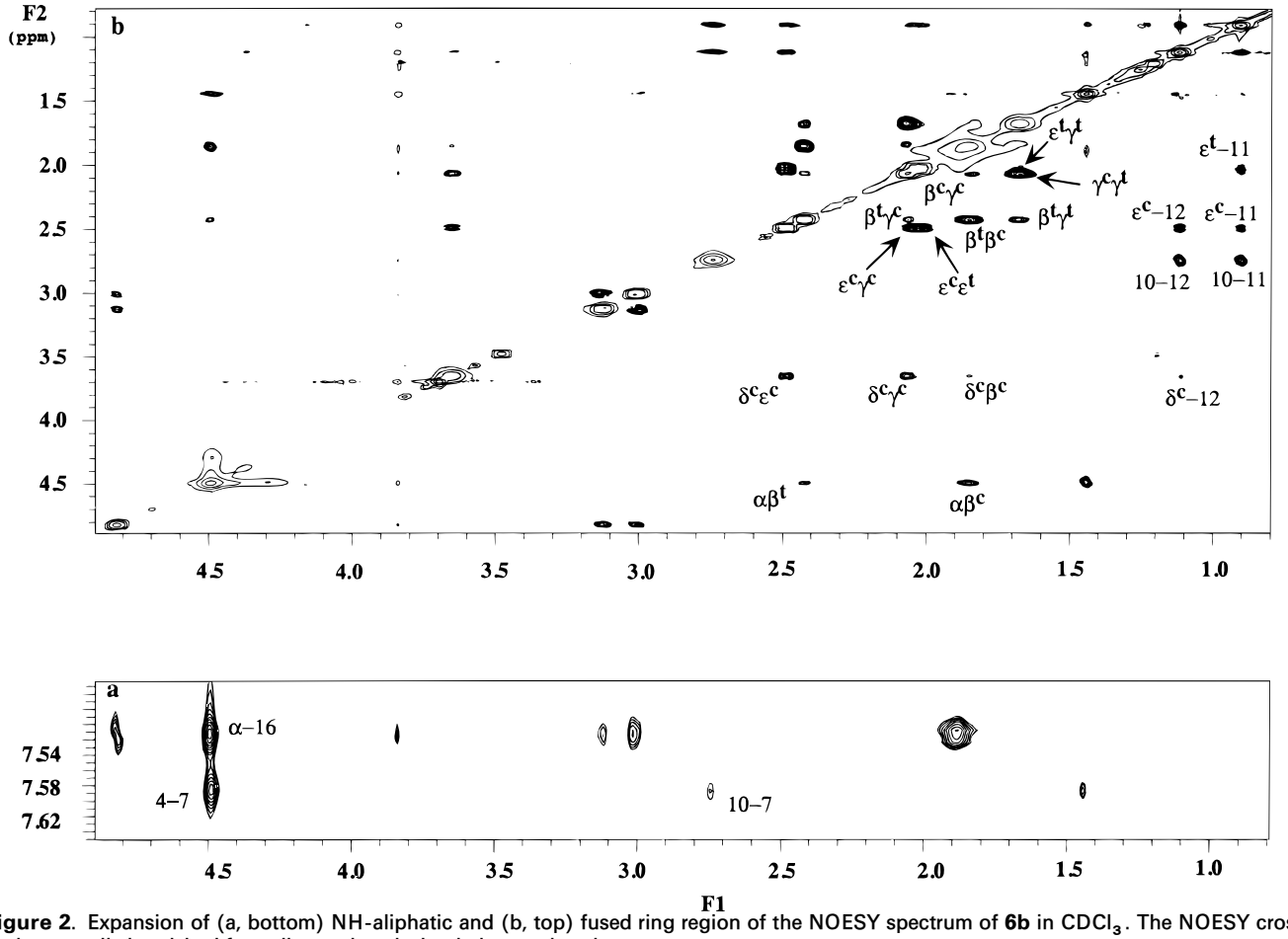
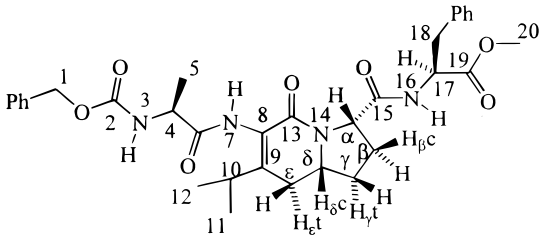


Figure 2. Expansion of (a, bottom) NH-aliphatic and (b, top) fused ring region of the NOESY spectrum of **6b** in $CDCl_3$. The NOESY cross peaks were distinguished from diagonal peaks by their negative character.



Scheme 3

Stereochemical and ring conformation assignment of the five-membered ring

Although the proton–proton connectivities of the proline ring could be established by COSY and HOHAHA experiments, the stereochemistry of C_δ relative to C_α and the stereochemistry of the individual proton resonances on C_β and C_γ remained unassigned. The stereochemistry of these protons was determined by an examination of vicinal proton–proton coupling constants (^3J) obtained by iterative spectral simulation (Fig. 3) and by 2D NOE measurements.

For the spectral simulation, estimates of the coupling constants were first obtained by selective irradiation of several specific regions in the double resonance experiment. Varian VNMR software was used to generate a simulated spectrum from the estimated coupling constants. The five-membered ring proline derivative was then treated as a six-spin system and as many resonances as possible were assigned to the observed spectrum. A least-squares iteration was performed to obtain the best-fit chemical shifts and coupling constants. These chemical shifts and coupling constants are reported in Tables 1 and 2. The coupling constants were then used to generate torsional angles $\chi(\alpha\beta)$, $\chi(\beta\gamma)$ and $\chi(\gamma\delta)$ by exploiting the angular dependence of the coupling constants with the Bystrov parameterization.[10] For example, the observed coupling constants between the proline α-proton and the β-methylene protons of $^3J(\alpha\text{–}\beta^c) = 8.8$ Hz and $^3J(\alpha\text{–}\beta^t) = 0.1$ Hz gave rise to dihe-

Table 1. Proton and carbon chemical shifts (ppm) of 6b in $CDCl_3$

	H-1	C-13
1	5.14	67.04
3	5.51	
4	4.49	50.77
5	1.44	19.23
7	7.59	
10	2.74	30.49
11	0.91	19.11
12	1.12	20.37
α	4.50	58.74
β	$\beta^t = 2.40$, $\beta^c = 1.88$	27.22
γ	$\gamma^t = 1.64$, $\gamma^c = 2.08$	31.61
δ	3.66	57.39
ε	$\varepsilon^t = 2.05$, $\varepsilon^c = 2.49$	29.03
16	7.51	
17	4.81	53.46
18	3.13, 3.01	37.89
20	3.70	52.30

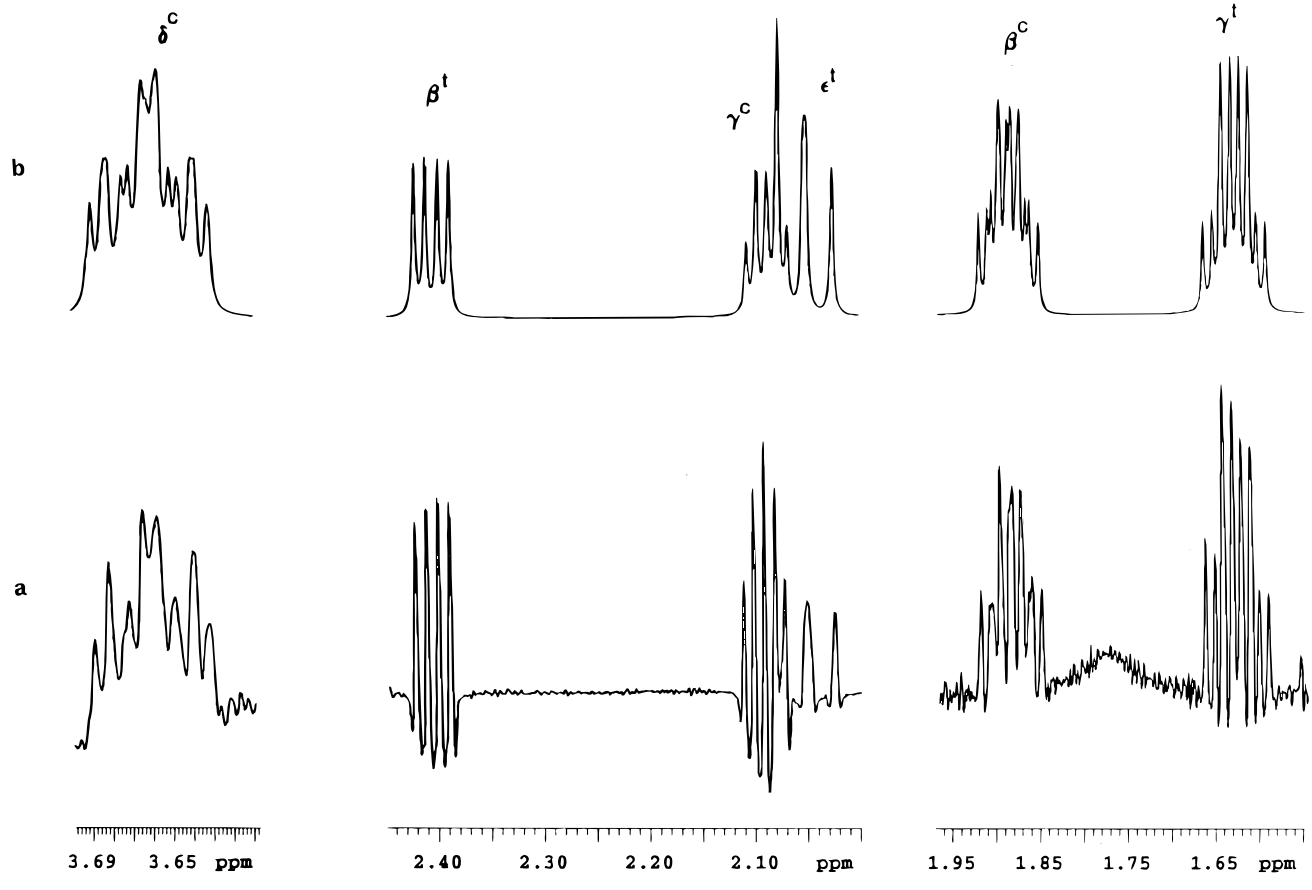


Figure 3. (a) Observed and (b) calculated spectra of the fused ring resonances.

Table 2. Vicinal J_{HH} values, torsional angles[a] (χ) at fused ring and Phe side-chain rotamer population for 6b

	$^3J_{HH}$ (Hz)	Torsional angle χ (°)	Rotamer
Ala	$J_{3,4} = 7.74$		
Fused ring	$J_{\alpha\beta^c} = 8.8$	15 (26)	
	$J_{\alpha\beta^t} = 0.1$	105 (92)	
	$J_{\beta^c\gamma^c} = 5.84$	40 (39)	
	$J_{\beta^c\gamma^t} = 12.55$	170 (160)	
	$J_{\beta^t\gamma^c} = 0.30$	85 (79)	
	$J_{\beta^t\gamma^t} = 6.23$	40 (40)	
	$J_{\gamma^c\delta^c} = 5.00$	40 (38)	
	$J_{\gamma^t\delta^c} = 11.50$	170 (160)	
	$J_{\delta^c\varepsilon^c} = 3.96$	60 (42)	
	$J_{\delta^c\varepsilon^t} = 15.22$	180 (160)	
Phe	$J_{16,17} = 7.90$		
	$J_{17,18} = 5.90, 7.12$		$g^- \approx 45\%$, $t \approx 35\%$, $g^+ \approx 20\%$

[a] Torsional angles were estimated from spin coupling constant ^3J; the values in parentheses are from energy-minimized conformation.

Table 3. Volume integrals for selected NOE cross peaks of 6b

Signal one	Signal two	NOE cross peak volume integral
H_α	H_{β^c}	−2.9
H_α	H_{β^t}	−1.2
H_{β^c}	H_{γ^c}	−1.8
H_{β^c}	H_{γ^t}	—
H_{γ^c}	H_{δ^c}	−3.1
H_{γ^t}	H_{δ^c}	—
H_{β^c}	H_{δ^c}	−0.78
H_{δ^c}	H_{ε^c}	−2.6
H_{γ^c}	H_{ε^c}	−4.5[a]
H_{γ^t}	H_{ε^t}	−6.4[a]
H_{10}	N_7H	−0.39
H_{ε^t}	H-11	−2.3
H_{δ^c}	H-12	−1.3

[a] Overlap with other resonance.

dral angles of 15° for $\chi(\alpha\beta^c)$ and −105° for $\chi(\alpha\beta^t)$ (Table 2). Hence the higher field β-resonance (β^c) was assigned as the methylene proton *cis* to the proline α-proton.

The observed coupling constants and dihedral angles for the α and β protons suggested that the proline ring had a predominately *endo* conformation.[11] The three dihedral angles $\chi(\beta^c\gamma^c)$, $\chi(\beta^t\gamma^t)$ and $\chi(\gamma^c\delta^c)$ were determined to be *ca.* −40° (with $^3J \approx 5.5$ Hz) and the dihedral angle $\chi(\beta^t\gamma^c)$ was determined to be *ca.* 85° [$^3J(\beta^t\text{–}\gamma^c) = 0.3$ Hz]. These angles again suggested that the proline ring was in an *endo* conformation.

Finally, the calculated dihedral angles for $\chi(\beta^c\gamma^t)$ and $\chi(\gamma^t\delta^c)$ were approximately −170° [with $^3J(\beta^c\text{–}\gamma^t) = 12.6$ Hz and $^3J(\gamma^t\text{–}\delta^c) = 11.5$ Hz]. The value obtained for these angles indicated that the fused proline ring possessed a puckered conformation in which the δ-proton had a *cis* orientation with respect to the proline α-proton. The stereochemistry of the bicyclic ring in **6b** was the same as that of the initial peptide building block **5b**.

The stereochemical assignments made above using the calculated coupling constants were confirmed using NOESY data (Table 3). Since the magnitude of an NOE signal is strongly dependent on the inter-proton distance, the NOE signal for vicinal protons on a five-membered ring that are *cis* to each other is normally larger than the NOE signal for vicinal protons on a five-membered ring that are *trans* to each other. This relationship has proven to be effective for the assignment of a variety of bicyclic lactam building blocks.[4a,6] For **6b** (Fig. 2), the proline α-proton showed a larger NOESY cross peak to the higher-field β-resonance [$d(\alpha\text{–}\beta^c)$ volume integral = −2.9] than to the lower field β-resonance [$d(\alpha\text{–}\beta^t)$, volume integral = −1.2]. Based on these data, the higher field β-resonance could be assigned as being *cis* to the α-proton. This assignment was in agreement with the dihedral angles $\chi(\alpha\beta^c)$ and $\chi(\alpha\beta^t)$ determined above. In a similar fashion, the γ-proton *cis* to the proline α-proton and the γ-proton *trans* to the proline α-proton were identified. In this case, $d(\beta^c\text{–}\gamma^c)$ led to a large cross peak (volume integral = −1.8) while $d(\beta^c\text{–}\gamma^t)$ was absent. With the *cis* and *trans* methylene protons at C_β and C_γ assigned, the stereochemistry at the bridgehead carbon (C_δ) could be determined. To this end, a strong NOESY cross peak was observed for $d(\delta^c\text{–}\gamma^c)$ (volume integral = −3.1), whereas no NOESY cross peak was observed for $d(\delta^c\text{–}\gamma^t)$. The bridgehead proton also showed a NOESY cross peak with the proton of the β-carbon that was known to be *cis* to the proline α-proton [$d(\delta^c\text{–}\beta^c)$/volume integral = −0.78]. In all cases, the NOESY data supported the stereochemical conclusions made using the calculated coupling constants from the NMR simulation.

A strong NOESY cross peak for $d(\delta^c\text{—}\varepsilon^c)$ (volume integral = −2.6) and a large calculated coupling constant for the interaction between $H_{\delta c}$ and $H_{\varepsilon t}$ ($J = 15.2$ Hz) suggested that the six-membered ring was in a half-chair conformation with *trans*, diaxial protons along the $C_\delta\text{–}C_\varepsilon$ bond. This conformation for the six-membered ring was also supported by the strong NOESY cross peaks observed for $d(\gamma^c\text{–}\varepsilon^c)$ (volume integral = −4.5) and $d(\gamma^t\text{–}\varepsilon^t)$ (volume integral = −6.4).

Backbone and side-chain conformations

It was found that the chemical shift difference for the β and γ geminal protons was fairly large: $\Delta\delta = 0.6$ ppm for the β methylene protons and $\Delta\delta = 0.4$ ppm for the γ methylene protons (Table 1). Generally, only a small chemical shift difference (less that 0.3 ppm) is observed for proline β and γ geminal protons in a linear peptide. The small difference normally observed has been attributed to the somewhat flexible heterocyclic proline ring and the flexible peptide backbone. Owing to this inherent flexibility, any anisotropy effects arising from the carboxyl group on the proline ring tend to be averaged out.[12] In **6b**, the large chemical shift difference observed for the β geminal protons can be accounted for by the more rigid conformation imposed on the backbone by the fused bicyclic ring system. Furthermore, the bulky isopropyl group and the double bond incorporated into

the six-membered lactam ring tend to limit the ring flexibility even more and force the carboxyl group at C-15 to point away from the ring system. As a consequence, $H_{\beta t}$ is fixed in the deshielding cone of the C-15 carbonyl. This leads to the strong non-equivalence of $H_{\beta t}$ and $H_{\beta c}$. This observation was consistent with the vicinal couplings $^3J(\alpha\text{–}\beta)$ and the NOESY cross peaks for $d\alpha\beta^t$ and $d\alpha\beta^c$ described above.

The NOESY cross peak between the methine proton on H-10 and the N_7H proton [$d(NH_7\text{–}N_{10})$, volume integral = −0.39] indicated that H-10 was pointing towards the peptide backbone and was in a *cis*- orientation with respect to the C-8—C-9 double bond (Table 3). This arrangement was confirmed by NOESY cross peaks between C-11 and C-12 methyl groups and the pseudoequatorial proton $H_{\varepsilon c}$ [$d(\varepsilon^c\text{–}H_{11})$ and $d(\varepsilon^c\text{–}H_{12})$, Table 3]. The C-11 methyl group also showed a NOESY cross peak with $H_{\varepsilon t}$ [$d(\varepsilon^t\text{–}H_{11})$, volume integral = −2.3] and the C-12 methyl group a NOESY cross peak with $H_{\delta c}$ [$d(\delta^c\text{–}H_{12})$, volume integral = −1.3], confirming the assignment of $H_{\varepsilon t}$ and $H_{\delta t}$ as being *trans* diaxial to each other. No cross peaks were observed between the C-11 methyl group and $H_{\delta c}$ or the C-12 methyl group and $H_{\varepsilon t}$.

The conformation of the peptide backbone was examined by the angular dependence of the $^3J(NH\text{–}\alpha H)$ coupling constants. A value of *ca.* 7.8 Hz for both of the $^3J(NH\text{–}\alpha H)$ coupling constants indicated that the peptide backbone was partially in an extended chain conformation in both of these regions.[13] This conclusion was supported by the strong sequential NOESY cross peaks for $d(\alpha\text{-}NH_{16})$ and $d(H_4\text{–}NH_7)$. The rotamer population for the Phe residue was calculated using the best-fit values of vicinal $^3J(H_{17}\text{–}H_{18})$.[14] *Gauche* (−) and *trans* rotamers constituted the most dominate populations (*ca.* 80%) (Table 2).

CONCLUSIONS

We have found that a combination of 2D-NOE data and coupling constants from molecular simulation can provide an excellent means for rapidly establishing the stereochemistry of a bicyclic lactam-based conformational constraint in a peptide fragment. This information is essential if the conformationally restricted peptide fragments are to serve as effective chemical probes for examining the relationship between the predicted and actual biological activity of peptide fragments.

Acknowledgements

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