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# Supporting Information

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for

# The Entropy Balance of Nostocyclopeptide Macrocyclization Analyzed by NMR

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## Methods

**1. NMR sample preparations.** For the preparation of the NMR samples, 3.0 mg of the linear peptide aldehydes[S1] (Scheme S1) were dissolved in 0.72 mL of a partially deuterated $H_3PO_4/KH_2PO_4$ buffer solution ($H_2O/D_2O$ 5:1) at pH 3.0. The macrocyclizations (Scheme S2) were carried out in the NMR tube. To the samples prepared as described above, solid $Na_2CO_3$ was added until pH reached about 7.0. After 20 min complete cyclization was observed in the $^1H$ NMR spectra. It should be noted that all pH and temperature changing experiments were carried out with just one same peptide sample of ncpA1 and ncpA2, respectively. This demonstrates that the systems can be modified repeatedly and in different ways without losing their reversibility.

**2. NMR measurements.** All measurements were performed on a Bruker Avance DRX 600 spectrometer with a 5 mm BBI probe head. Water suppression was achieved by excitation sculpting with gradients (double watergate DPFGSE sequence).[S2] Homonuclear 2D spectra (TOCSY, ROESY) were recorded in the phase-sensitive mode as data matrices of 512 ($t_1$) real x 2048 ($t_2$) complex data points; 20 to 32 scans (TOCSY) and 16 to 32 scans (ROESY), respectively, were used per $t_1$ increment. The used spectral widths were 6010 Hz in each

dimension for the TOCSY experiments and 7210 Hz (lin-ncpA2: 6600 Hz) in each dimension for the ROESY experiments, respectively. Mixing times of 100 ms (TOCSY) and 300 ms (ROESY) were applied. Heteronuclear 2D HSQC experiments were performed in the phase-sensitive mode with data matrices of 256 ($t_1$, $^{13}$C) real x 2048 ($t_2$, $^{1}$H) complex data points and 80 scans per $t_1$ increment. The HSQC measurement of ncpA1 was performed using a data matrix of 512 ($t_1$, $^{13}$C) real x 2048 ($t_2$, $^{1}$H) complex data points with 26 scans per $t_1$ increment. The used spectral width were 6600 ($^{1}$H) / 22640 Hz ($^{13}$C) for the ncpA1 species and 8010 ($^{1}$H) / 12070 Hz ($^{13}$C) for the ncpA2 species, respectively. All data were recorded and analyzed using Bruker TopSpin software. The spectra were calibrated on the $H_2O$ resonance (4.80 ppm at 300 K).[S3] The fully assigned $^{1}$H NMR spectra are depicted in Figures S1 and S2, and all proton chemical shifts are listed in Tables S1 and S2.

**3. Temperature dependence of macrocyclization equilibria.** The percentages of linear and cyclic species were determined by comparing the integrals of well-separated signals from $^{1}$H NMR spectra between 290 and 330 K (10 K steps). After every temperature change the system was allowed to equilibrate for 1 h. For both peptides the measurements were performed at pH 5.2. In order to ensure consistent analyses, $C_\beta$-H signals in the same spectral region were used in both cases. All spectra and percentages are depicted in Figure S3.

**4. Calculation of the macrocyclization entropies.** According to the *van't Hoff* equation

$$\frac{d \ln K}{d 1/T} = -\frac{\Delta H}{R} \qquad \text{with } K = \frac{\%\,(cycl)}{\%\,(lin)} \tag{1}$$

–lnK was plotted versus 1/T and fitted linear (Figure S4 A, B). The linear fit gradients gave cyclization enthalpies of $\Delta H = +19.3$ kJ mol$^{-1}$ (ncpA1) and $\Delta H = +16.4$ kJ mol$^{-1}$ (ncpA2), respectively. Linear fits of the plots of percentage vs. temperature (Figure S4 C, D) give the temperatures with $K = 1$ and $\Delta G = 0$, which allow to calculate the entropy balances of macrocyclization are calculated by use of the Gibbs-Helmholtz equation

$$\Delta G = \Delta H - T\Delta S \tag{2}$$

For the ncpA1 equilibrium $K = 1$ at 321.1 K, and for the ncpA2 equilibrium $K = 1$ at 319.4 K. With the values for $\Delta H$ and setting $\Delta G = 0$ at the respective temperatures, the *Gibbs-Helmholtz* equation gives the entropy balances of macrocyclization:

$\Delta S = +59.9$ J K$^{-1}$mol$^{-1}$ = + 14.3 cal K$^{-1}$mol$^{-1}$ (ncpA1)

$\Delta S = +51.4$ J K$^{-1}$mol$^{-1}$ = + 12.3 cal K$^{-1}$mol$^{-1}$ (ncpA2)

These values represent the total entropy changes occurring by cyclization with the two released water molecules included. In this context, the relative solvation of linear and cyclic species also influences the macrocyclization entropy as the reduced mobility of bound solvent molecules contributes to the total entropy.[S4-S6] In a first approximation, disregarding changes in the solvation sphere, the main factors influencing the observed temperature dependence are the number of molecules involved in the reaction and the conformational restriction of the peptide chain. The nostocyclopeptides keep their positive charge upon cyclization and, as the two polar end groups already interact with each other in the linear peptides, the condensation of the aldehyde hydrate with the amine should not have great impact on hydration. This conception is supported the mostly only minor changes of $^{1}$H chemical shifts from linear to cyclic peptides in the middle sections (Tables S1 and S2), and should consequently allow to neglect the changes of hydration upon nostocyclopeptide macrocyclization and therewith the involved contribution to the overall entropy balance. With this approximation of a constant number of molecules in the hydrating shell, a total release of two water molecules upon the cyclization process can be assumed. Hence, as the cyclizations are accompanied by an entropy gain, the absolute values of cyclization entropy should be determined by the water release and not by the loss of degrees of freedom within the backbone.

**5. pH dependence of macrocyclization equilibria.** For the examination of the pH dependence of the ncpA2 equilibria, step-by-step increase of pH value (3.0 → 6.5) was achieved by addition of solid $Na_2CO_3$ directly into the NMR tube, and spectra were recorded 5-15 min after pH change. Subsequent acidification (pH 6.5 → 4.0) was carried out with the same sample by addition of aqueous $H_3PO_4$. All percentages are listed in Table S3 and in Figure 2 of the main section. The percentages were determined by comparing the integrals ($^{1}$H NMR spectra) of well-separated amide proton signals.

**6. NMR parameters.** Temperature gradients (Table S4) were determined by recording $^{1}$H NMR spectra at different temperatures from 290 to 320 K at pH 3.0 (linear peptides) and pH 7.0 (cyclic peptides). The calculation of the rotamer distributions (Table S5) was carried out for the Tyr side chain in linear and cyclic ncpA1 and ncpA2 species as well as for the Phe side chain in cyclic ncpA2. For the $C_\alpha$-H/$C_\beta$-H scalar coupling of aromatic side chains a minimum value of 3.55 Hz ($^3J_{gauge}$) and maximum value of 13.90 Hz ($^3J_{gauge}$) was assumed.[S7] With the $^3J$ coupling constants of the $C_\beta$-$H_{proS}$ and $C_\beta$-$H_{proS}$ protons, the populations *p* of *gauge-trans* (*gt*), *trans-gauge* (*tg*), and *gauge-gauge* (*gg*) rotamers are calculated using a three-equation system:

$$p_{gt} = \frac{^3J_{proS} - ^3J_{gauge}}{^3J_{trans} - ^3J_{gauge}} \quad (3)$$

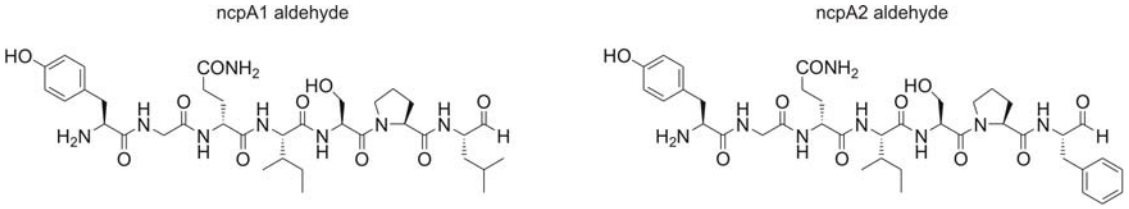
$$p_{tg} = \frac{^3J_{proR} - ^3J_{gauge}}{^3J_{trans} - ^3J_{gauge}} \quad (4)$$

$$p_{gg} = 1 - p_{gt} - p_{tg} \quad (5)$$

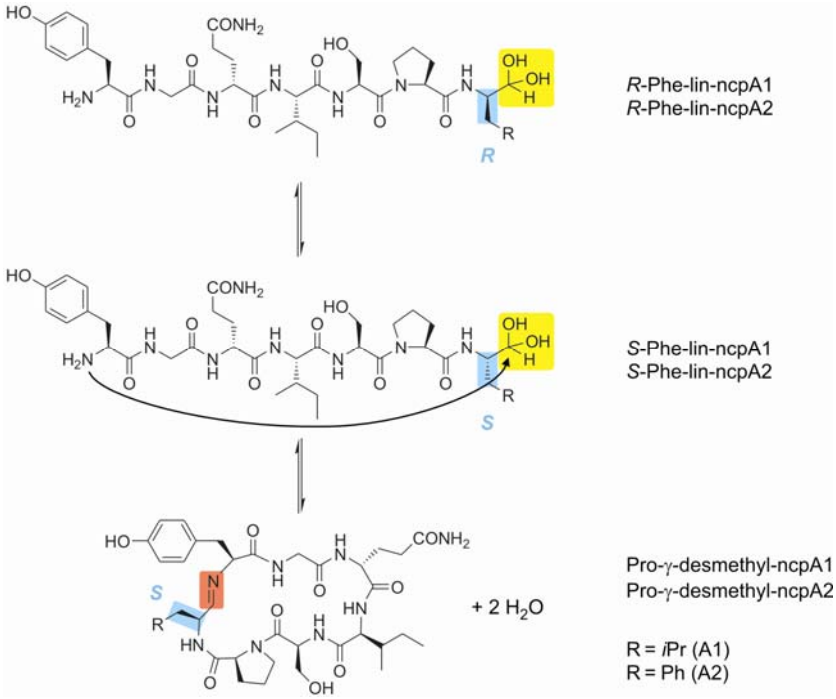
**7. NOE-based molecular modeling of *S*-Phe-lin-ncpA2 and ncpA2.** Interproton-distance restraints were derived from homonuclear NOESY experiments and classified into three categories strong (~ 2.2 Å), medium (~ 3 Å), and weak (~ 4 Å), serving as distance restraints for the structure calculations.[S8] The selected NOE contacts and the NOE intensity derived distances (target dist.) are given in Tables S6 and S7. The molecular dynamics (MD) simulation resulted in distances listed in the MD columns. For all calculations the program package *HyperChem* [S9] was used using MM+ force field without explicit water included, and no explicit parametrization was used. Ten snapshots from the 10 x 10 ps molecular dynamics simulations (step size 1 fs, 300 K) are shown in Figures S5 (*S*-Phe-lin-ncpA2) and S6 (ncpA2).

The ten snapshots were averaged and the resulting structures were subjected to energy minimization (EM) after the NOE constraints had been removed, leading to the structures as shown in Figure 3A, main section. The distances resulting from the energy minimization are shown in the EM columns (Tables S6 and S7). For the MD and EM experiments, all differences to the target distances are listed. The last columns show all changes in distances from the NOE-derived structures to the energy minimized structures (Diff. EM–MD). Only few greater differences before and after energy minimization were observed. These mainly resulted from distances with the terminal and thus more mobile Tyr or Phe involved. For the peptide backbone and the other side chains, differences are small, which speaks for the plausibility of the structures obtained.

## Schemes, Figures, and Legends




**Scheme S1.** Synthesized linear peptide aldehydes. The linear cyclization precursors were synthesized on solid phase as described in ref. S1. For synthetic reasons, the naturally occurring (2*S*,4*S*)-methylproline was replaced by proline.




**Scheme S2.** The nostocyclopeptides' epimerization and cyclization equilibria. In aqueous solution the linear cyclization precursors predominantly exist as aldehyde hydrates (>95%, yellow) and epimerize at the stereo center of the C terminal amino acid (blue). Only the *S*-configurated linear peptides (*S*-Phe-lin-ncpA1, *S*-Phe-lin-ncpA2) are able to cyclize, yielding stereochemically pure *S*-configurated cyclopeptides ncpA1 and ncpA2, respectively, with an imine moiety (red).

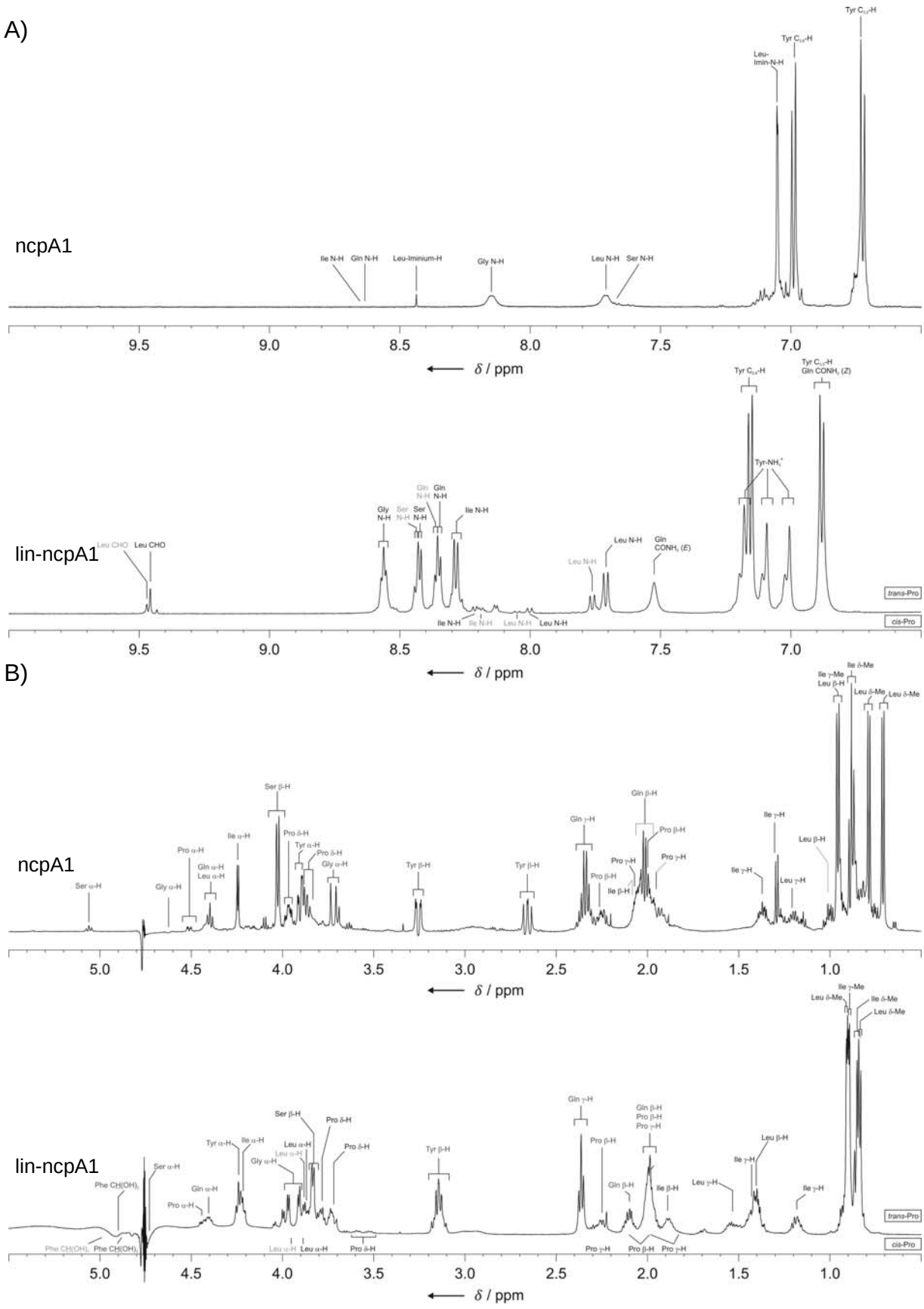



**Figure S1.** Assigned $^1$H NMR spectra (watergate, 600 MHz, 300 K, $H_2O/D_2O$ 5:1) of lin-ncpA1 (pH 3.0) and ncpA1 (pH 7.0). A) low field range. B) high field range. In the spectra of lin-ncpA1, *R* epimer signals are designated grey and *S* epimer signals are designated black. Some signals of the linear *cis*-Pro peptides are also assigned.

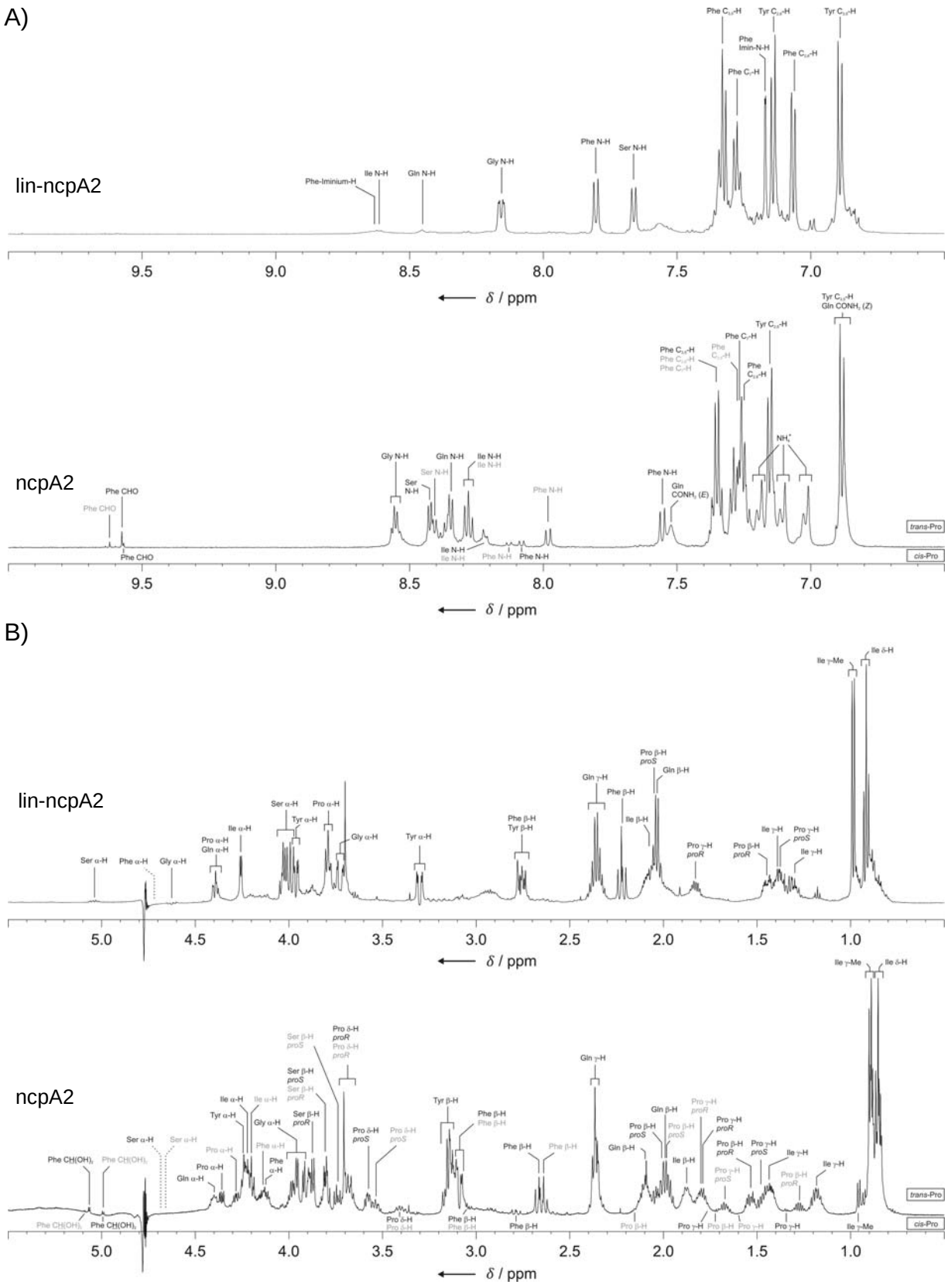



**Figure S2.** Assigned $^{1}$H NMR spectra (watergate, 600 MHz, 300 K, $H_2O/D_2O$ 5:1) of lin-ncpA2 (pH 3.0) and ncpA2 (pH 7.0). A) low field range. B) high field range. In the spectra of lin-ncpA2, *R* epimer signals are designated grey and *S* epimer signals are designated black. Some signals of the linear *cis*-Pro peptides are also assigned.

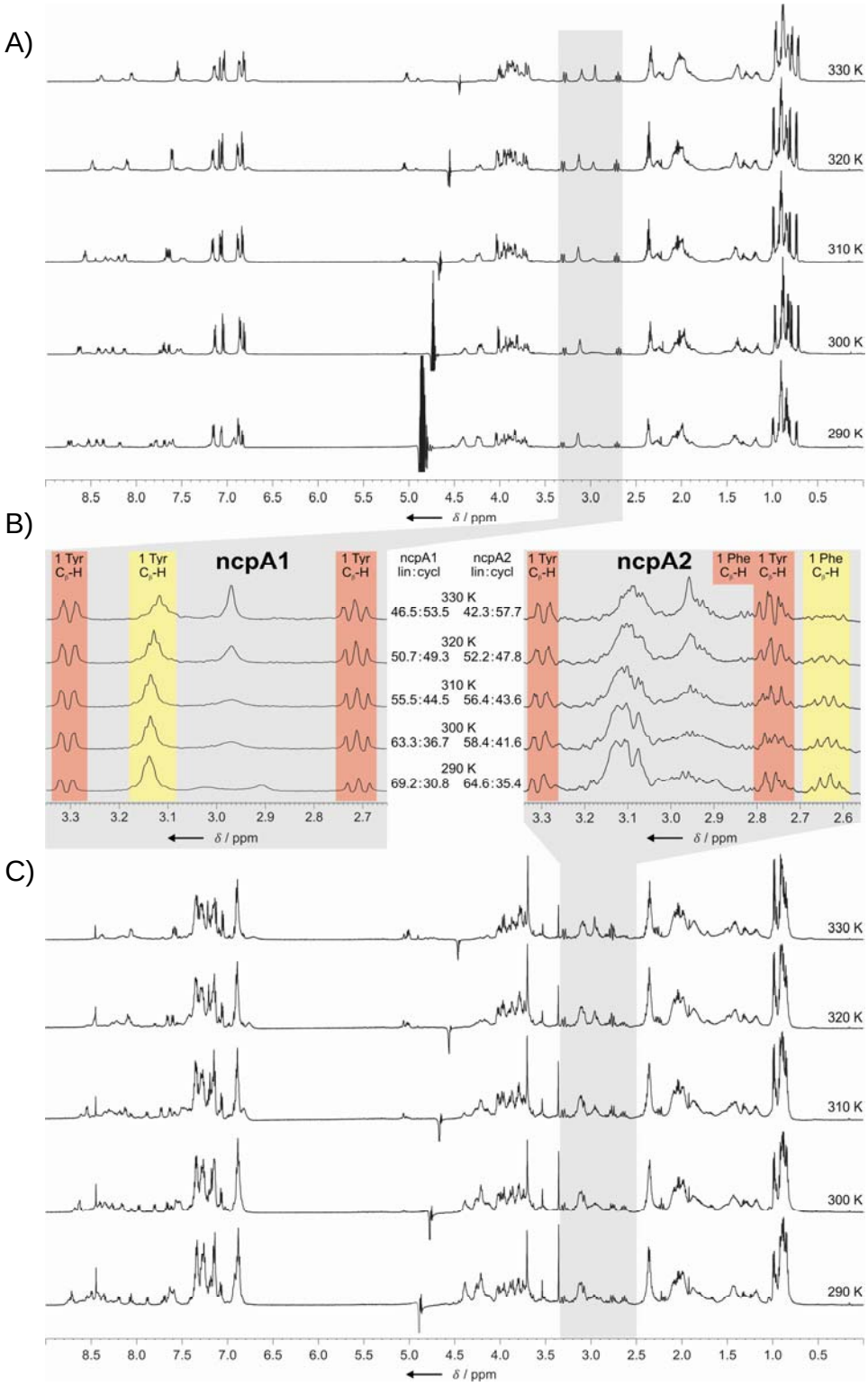



**Figure S3.** Temperature dependence of the nostocyclopeptides' cyclization equilibria. $^{1}$H NMR spectra (watergate, 600 MHz, $H_2O/D_2O$ 5:1, pH 5.2) of the equilibrating nostocyclopeptides between 290 and 320 K (10 K steps) are shown. A) Complete spectra of lin-ncpA1/ncpA1 equilibrium system. B) Enlargement of spectral sections containing the $C_\beta$-H signals used for determination of linear/cyclic ratios which are also depicted. Signals resulting from linear species are marked yellow and signals resulting from cyclic species are marked red, respectively. C) Complete spectra of lin-ncpA2/ncpA2 equilibrium system.

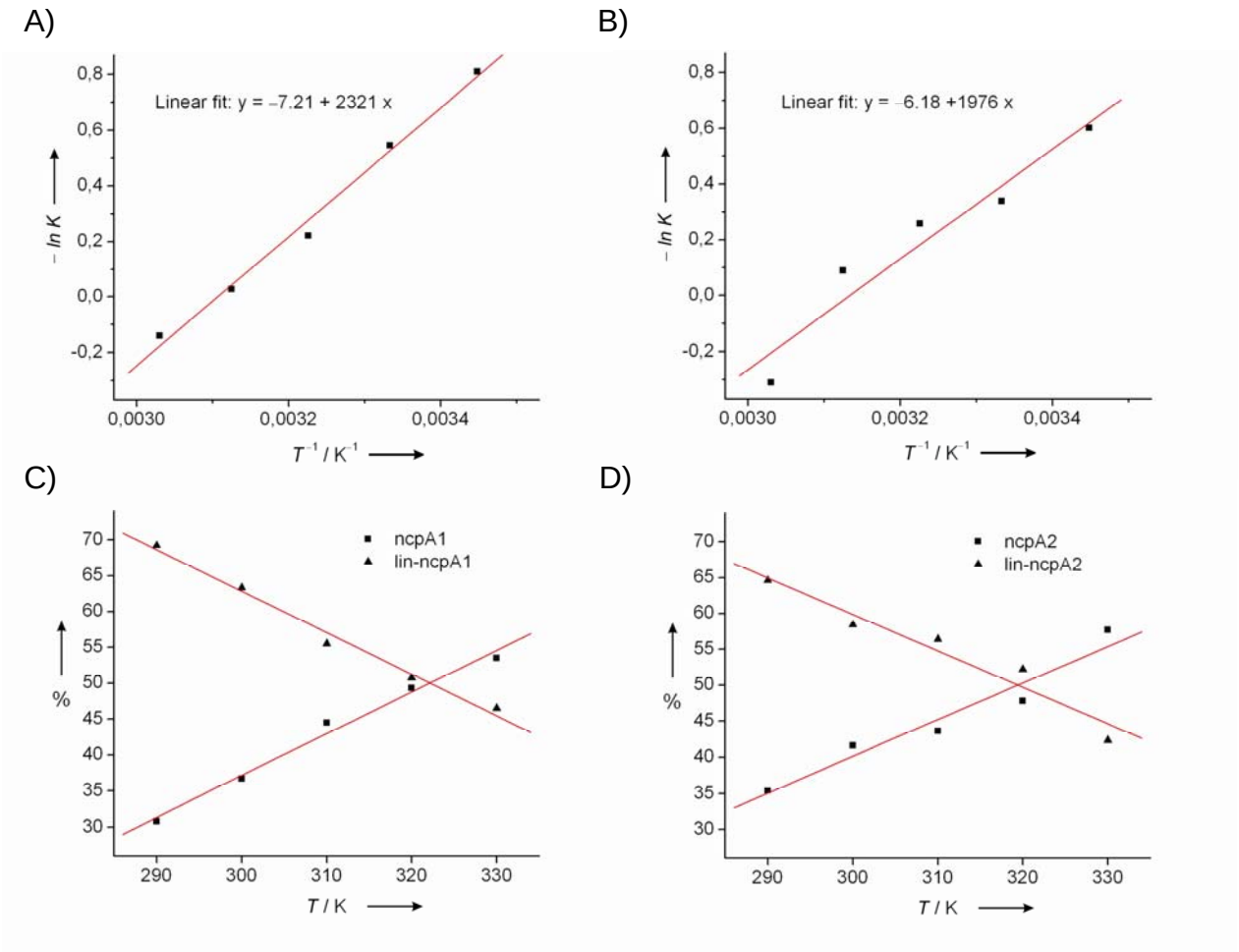



**Figure S4.** Calculation of the cyclization entropy balances. A) *van't Hoff* plot of ncpA1 equilibrium. B) *van't Hoff* plot of ncpA2 equilibrium. C) Determination of the temperature with K = 1 for ncpA1 equilibrium. D) Determination of the temperature with K = 1 for ncpA2 equilibrium. Please see Methods section 4 for further information.

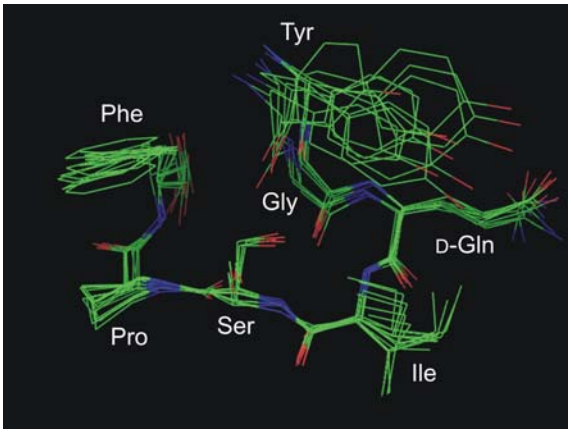



**Figure S5.** Overlay of ten snap shots (taken in 10 ps steps) of the Molecular Dynamics simulation of *S*-Phe-lin-ncpA2.

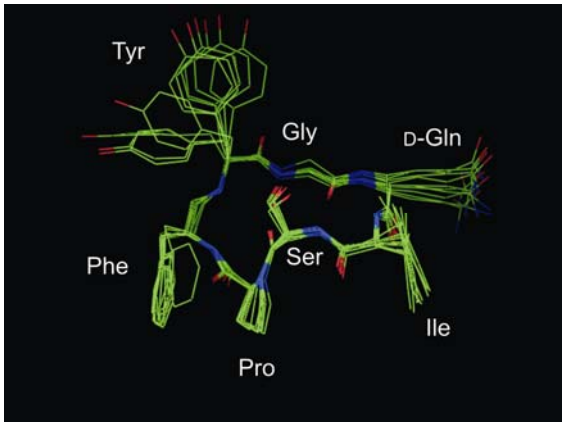



**Figure S6.** Overlay of ten snap shots (taken in 10 ps steps) of the Molecular Dynamics simulation of ncpA2.

## Tables

**Table S1.** $^{1}$H chemical shifts (in ppm) of ncpA1 species.

| Position | | *R*-Phe-lin-ncpA1 | *S*-Phe-lin-ncpA1 | ncpA1 |
|---|---|---|---|---|
| **Tyr** | $NH_3^+$ | 7.02-7.20 m | 7.02-7.20 m | -- |
| | $C_\alpha$-H | 4.24 *t* (7.3) | 4.24 *t* (7.3) | 3.91 *dd* (3.4, 11.6) |
| | $C_\beta$-H | 3.12 *dd* (7.3, 14.1) | 3.12 *dd* (7.3, 14.1) | 2.76 *dd* (11.6, 14.2) |
| | | 3.17 *dd* (7.3, 14.1) | 3.17 *dd* (7.3, 14.1) | 3.30 *dd* (3.4, 14.2) |
| | $C_{2,6}$-H | 7.15 *d* (8.6) | 7.15 *d* (8.6) | 7.01 *d* (8.6) |
| | $C_{3,5}$-H | 6.88 *d* (8.6) | 6.88 *d* (8.6) | 6.75 *d* (8.6) |
| | OH | invisible | invisible | invisible |
| **Gly** | NH | 8.57 *t* (6.0) | 8.57 *t* (6.0) | 8.16 *dd* (3.3,10.0) |
| | $C_\alpha$-H | 3.90 *dd* (6.0, 16.7) | 3.90 *dd* (6.0, 16.7) | 3.73 *dd* (3.3, 17.2) |
| | | 3.98 *dd* (6.0, 16.7) | 3.98 *dd* (6.0, 16.7) | 4.63 [1] |
| **Gln** | NH | 8.36 *d* (7.2) | 8.35 *d* (7.2) | 8.63 *d* (5.9) |
| | $C_\alpha$-H | 4.41 *m* | 4.41 *m* | 4.39 *m* |
| | $C_\beta$-H | 1.99 *m* | 1.99 *m* | 2.04 *pq* (8.1) |
| | | 2.11 *m* | 2.11 *m* | |
| | $C_\gamma$-H | 2.37 *t* (7.6) | 2.37 *t* (7.6) | 2.36 *m* |
| | $CONH_2$ (*Z*) | 6.87 *br s* | 6.87 *br s* | 6.88 *br s* |
| | $CONH_2$ (*E*) | 7.53 *br s* | 7.53 *br s* | 7.57 *br s* |
| **Ile** | NH | 8.29 *d* (8.2) | 8.29 *d* (8.2) | 8.65 *d* (7.7) |
| | $C_\alpha$-H | 4.22 *t* (8.0) | 4.22 *t* (8.0) | 4.25 *m* |
| | $C_\beta$-H | 1.89 *m* | 1.89 *m* | 2.08 *m* |
| | γ-Me | 0.90 *d* (7.0) | 0.90 *d* (7.0) | 0.99 *d* (7.2) |
| | $C_\gamma$-H | 1.18 *m* | 1.18 *m* | 1.30 *m* |
| | | 1.43 *m* | 1.43 *m* | 1.39 *ddd* (4.6, 7.8, 13.6) |
| | $C_\delta$-H | 0.86 *t* (7.6) | 0.86 *t* (7.6) | 0.91 *t* (7.7) |
| **Ser** | NH | 8.44 *d* (6.6) | 8.42 *d* (6.6) | 7.66 *d* (8.7) |
| | $C_\alpha$-H | 4.74 [1] | 4.74 [1] | 5.06 *pq* (8.6) |
| | $C_\beta$-H | 3.83 *m* | 3.83 *m* | 4.03 *d* (8.1) |
| | OH | invisible | invisible | invisible |
| **Pro** | $C_\alpha$-H | 4.45 *d* (4.1) | 4.45 *d* (4.5) | 4.52 *dd* (3.5, 9.5) |
| | $C_\beta$-H | 1.99 *m* | 1.99 *m* | 2.00 *m* (pro*R*) |
| | | 2.25 *m* | 2.25 *m* | 2.28 *m* (pro*S*) |
| | $C_\gamma$-H | 1.99 *m* | 1.99 *m* | 2.07 *m* (pro*R*) |
| | | | | 1.95 *m* (pro*S*) |
| | $C_\delta$-H | 3.73 *m* | 3.73 *m* | 3.88 *m* (pro*R*) |
| | | 3.79 *m* | 3.79 *m* | 3.98 *m* (pro*S*) |
| **Leu** | NH | 7.76 *d* (9.6) | 7.71 *d* (9.6) | 7.73 *d* (9.1) |
| | $C_\alpha$-H | 3.90 *m* | 3.87 *m* | 4.43 *m* |
| | $C_\beta$-H | 1.40 *m* | 1.41 *m* | 0.98 *m* |
| | | | | 1.03 *m* |
| | $C_\gamma$-H | 1.50 *m* | 1.55 *m* | 1.23 *m* |
| | $C_\delta$-H | 0.84 *d* (6.9) | 0.84 *d* (6.9) | 0.74 *d* (7.2) |
| | | 0.91 *d* (6.9) | 0.91 *d* (6.9) | 0.82 *d* (7.2) |
| | CHO | 9.57 *s* | 9.62 *s* | -- |
| | $C\underline{H}(OH)_2$ | 4.93 [1] | 4.90 [1] | -- |
| | $CH(O\underline{H})_2$ | invisible | invisible | -- |
| | CH=N | -- | -- | 7.07 *d* (2.0) |

1) No coupling constants were obtained due to weak or broad signals.

**Table S2.** $^{1}$H chemical shifts (in ppm) of ncpA2 species.

| Position | | *R*-Phe-lin-ncpA2 | *S*-Phe-lin-ncpA2 | ncpA2 |
|---|---|---|---|---|
| **Tyr** | $NH_3^+$ | 7.01-7.20 m | 7.01-7.20 m | -- |
| | $C_\alpha$-H | 4.24 *t* (7.3) | 4.24 *t* (7.3) | 3.96 *dd* (3.6, 11.3) |
| | $C_\beta$-H | 3.12 *dd* (7.2, 14.1) | 3.12 *dd* (7.2, 14.1) | 2.76 *dd* (11.2, 14.1) |
| | | 3.16 *dd* (7.2, 14.1) | 3.16 *dd* (7.2, 14.1) | 3.30 *dd* (3.5, 14.1) |
| | $C_{2,6}$-H | 7.15 *d* (8.6) | 7.15 *d* (8.6) | 7.14 *d* (8.6) |
| | $C_{3,5}$-H | 6.88 *d* (8.6) | 6.88 *d* (8.6) | 6.89 *d* (8.6) |
| | OH | invisible | invisible | invisible |
| **Gly** | NH | 8.56 *dd* (6.0, 6.4) | 8.56 *dd* (6.0, 6.4) | 8.16 *dd* (3.3,10.0) |
| | $C_\alpha$-H | 3.91 *m* | 3.91 *m* | 3.73 *dd* (3.3, 17.4) |
| | | 3.96 *m* | 3.96 *m* | 4.63 [1)] |
| **Gln** | NH | 8.35 *d* (7.2) | 8.35 *d* (7.2) | 8.45 [1)] |
| | $C_\alpha$-H | 4.40 *m* | 4.40 *m* | 4.39 *t* (8.0) |
| | $C_\beta$-H | 1.99 *m* | 1.99 *m* | 2.04 *m* |
| | | 2.11 *m* | 2.11 *m* | |
| | $C_\gamma$-H | 2.37 *t* (7.6) | 2.37 *t* (7.6) | 2.36 *m* |
| | $CONH_2$ (*Z*) | 6.87 *br s* | 6.87 *br s* | 6.88 *br s* |
| | $CONH_2$ (*E*) | 7.52 *br s* | 7.52 *br s* | 7.57 *br s* |
| **Ile** | NH | 8.29 *d* (8.1) | 8.29 *d* (8.1) | 8.61 [1)] |
| | $C_\alpha$-H | 4.20 *t* (8.1) | 4.22 *t* (8.1) | 4.26 *d* (4.6) |
| | $C_\beta$-H | 1.88 *m* | 1.88 *m* | 2.06 *m* |
| | γ-Me | 0.89 *d* (7.0) | 0.89 *d* (7.0) | 0.99 *d* (7.1) |
| | $C_\gamma$-H | 1.18 *m* | 1.18 *m* | 1.30 *ddd* (7.6, 9.7, 13.5) |
| | | 1.43 *m* | 1.43 *m* | 1.39 *ddd* (4.6, 7.6, 13.5) |
| | $C_\delta$-H | 0.85 *t* (7.5) | 0.85 *t* (7.5) | 0.92 *t* (7.6) |
| **Ser** | NH | 8.40 *d* (6.7) | 8.42 *d* (6.4) | 7.66 *d* (8.9) |
| | $C_\alpha$-H | 4.66 *m* | 4.69 *m* | 5.04 *ddd* (6.7, 8.9, 9.6) |
| | $C_\beta$-H | 3.74 *dd* (7.3, 11.0) (pro*R*) | 3.81 *dd* (7.5, 11.0) (pro*R*) | 3.99 *dd* (9.6, 10.6) |
| | | 3.80 *dd* (7.5, 11.0) (pro*S*) | 3.88 *dd* (6.4, 11.0) (pro*S*) | 4.03 *dd* (6.7, 10.6) |
| | OH | invisible | invisible | invisible |
| **Pro** | $C_\alpha$-H | 4.28 *d* (9.8) | 4.36 *d* (9.6) | 4.40 *d* (3.7, 9.1) |
| | $C_\beta$-H | 1.27 *m* (pro*R*) | 1.53 *m* (pro*R*) | 1.45 *m* (pro*R*) |
| | | 1.98 *m* (pro*S*) | 2.03 *m* (pro*S*) | 2.05 *m* (pro*S*) |
| | $C_\gamma$-H | 1.80 *m* (pro*R*) | 1.79 *m* (pro*R*) | 1.83 *m* (pro*R*) |
| | | 1.66 *m* (pro*S*) | 1.47 *m* (pro*S*) | 1.38 *m* (pro*S*) |
| | $C_\delta$-H | 3.68 *m* (pro*R*) | 3.67 *m* (pro*R*) | 3.79 *m* |
| | | 3.53 *m* (pro*S*) | 3.57 *m* (pro*S*) | |
| **Phe** | NH | 7.98 *d* (9.8) | 7.55 *d* (9.6) | 7.80 *d* (9.3) |
| | $C_\alpha$-H | 4.15 *m* | 4.13 *m* | 4.72 [1)] |
| | $C_\beta$-H | 2.64 *m* | 2.66 *m* (pro*R*) | 2.22 *dd* (10.7, 14.2) (pro*R*) |
| | | 3.09 *dd* (4.7, 14.5) | 3.09 *dd* (4.7, 14.5) (pro*S*) | 2.76 *dd* (4.9, 14.2) (pro*S*) |
| | $C_{2,6}$-H | 7.35 *d* (7.5) | 7.25 *d* (7.5) | 7.07 *m* |
| | $C_{3,5}$-H | 7.29 *t* (7.5) | 7.35 *t* (7.5) | 7.33 *m* |
| | $C_4$-H | 7.35 *d* (7.5) | 7.28 *d* (7.5) | 7.27 *m* |
| | CHO | 9.57 *s* | 9.62 *s* | -- |
| | $CH\underline{H}(OH)_2$ | 5.07 *d* (4.3) | 4.99 *d* (4.9) | -- |
| | $CH(O\underline{H})_2$ | invisible | invisible | -- |
| | CH=N | -- | -- | 7.17 *d* (2.0) |

1) No coupling constants were obtained due to weak or broad signals.

**Table S3.** Percentages of linear and cyclic ncpA2 species in dependence of pH value and time.

| pH, time | *R*-Phe-lin-ncpA2 | *S*-Phe-lin-ncpA2 | ncpA2 |
|---|---|---|---|
| pH 3.0 | 40 | 60 | 0 |
| pH 4.5 | 37 | 35 | 28 |
| pH 5.5 | 32 | 20 | 48 |
| pH 6.0 | 20 | 10 | 70 |
| pH 6.5, 15 min | 16 | 0 [1)] | 84 |
| pH 6.5, 64 h | 14 | 0 [1)] | 86 |
| pH 6.5, 20 d | 10 | 0 [1)] | 90 |
| pH 6.5, 21 d | 9 | 0 [1)] | 91 |
| pH 6.0 | 9 [2)] | 10 | 81 |
| pH 5.0 | 13 | 20 | 67 |
| pH 4.0, 10 min | 14 | 27 | 59 |
| pH 4.0, 3.5 h | 17 | 27 | 56 |
| pH 4.0, 5 d | 29 | 32 | 39 |
| pH 4.0, 12 d | 34 | 34 | 32 |

1) Percentage of *S*-Phe-lin-ncpA2 < 5 % (not detectable)

2) Percentage of *R*-Phe-lin-ncpA2 was assumed not to have changed significantly with respect to the preceding measurement (weak signals only allowed determination of total amount of both linear species).

**Table S4.** Temperature gradients (in ppb/K) of linear and cyclic ncpA1 and ncpA2 species.

| position | *R*-Phe-lin-ncpA1 | *S*-Phe-lin-ncpA1 | ncpA1 | *R*-Phe-lin-ncpA2 | *S*-Phe-lin-ncpA2 | ncpA2 |
|---|---|---|---|---|---|---|
| Gly-NH | – 6.8 | – 6.8 | – 2.8 | – 6.7 | – 6.7 | – 3.6 |
| D-Gln-NH | – 7.3 | – 7.1 | – 7.9 | – 7.5 | – 7.1 | -- [1)] |
| Ile-NH | – 8.5 | – 8.5 | – 8.6 | – 8.3 | – 8.4 | -- [1)] |
| Ser-NH | – 9.3 | – 9.1 | – 2.9 | – 8.9 | – 9.3 | – 3.1 |
| Leu-NH | – 7.8 | – 7.9 | – 5.8 | -- | -- | -- |
| Phe-NH | -- | -- | -- | – 8.8 | – 6.1 | – 7.5 |

1) No data were obtained due to weak signals.

**Table S5.** Rotamer distributions (*gt* : *tg* : *gg*, in %) of aromatic ncpA1 and ncpA2 side chains. For scalar coupling constants see Table S1 and S2.

| position | *R*-Phe-lin-ncpA1 | *S*-Phe-lin-ncpA1 | ncpA1 | *R*-Phe-lin-ncpA2 | *S*-Phe-lin-ncpA2 | ncpA2 |
|---|---|---|---|---|---|---|
| Tyr | 1) | 1) | 0 : 78 : 22 | 1) | 1) | 0 : 74 : 26 |
| Phe | -- | -- | -- | 2) | 2) | 13 : 69 : 18 |

1) Both protons showed average coupling constants of 7.3 Hz (ncpA1) and 7.2 Hz (ncpA2), respectively, which results from unhindered side chain mobility with equal populations of all three rotamers.

2) Only the pro*S* coupling constant could be determined, which only differs in 0.2 Hz from the respective value of the cyclic species. Thus, similar rotamer populations as in nspA2 can be assumed.

**Table S6.** NOE restraints and acquired distances for *S*-Phe-lin-ncpA2. Please see Methods section 7 for explanations.

| NOE | target dist. | MD | Diff. | EM | Diff. | Diff. EM–MD |
|---|---|---|---|---|---|---|
| Phe $C_\beta$-$H^{pro R}$ / Phe C<u>H</u>$(OH)_2$ | 2.50 | 2.40 | – 0.10 | 2.65 | 0.15 | 0.25 |
| Phe $C_\beta$-$H^{pro S}$ / Phe C<u>H</u>$(OH)_2$ | 2.50 | 3.00 | 0.30 | 3.50 | 1.00 | 0.50 |
| Phe $C_\alpha$-H / Phe C<u>H</u>$(OH)_2$ | 3.00 | 3.00 | 0.00 | 3.00 | 0.00 | 0.00 |
| Phe $C_{2,6}$-H / Pro $C_\beta$-$H^{pro R}$ | 3.00 | 3.50 | 0.50 | 3.20 | 0.20 | – 0.30 |
| Phe $C_{3,5}$-H / Pro $C_\delta$-$H^{pro S}$ | 3.00 | 2.60 | – 0.40 | 4.15 | 1.15 | 1.55 |
| Phe NH / Phe $C_\beta$-$H^{pro R}$ | 2.50 | 2.65 | 0.15 | 2.65 | 0.15 | 0.00 |
| Phe NH / Phe C<u>H</u>$(OH)_2$ | 3.00 | 3.10 | 0.10 | 2.60 | – 0.40 | – 0.50 |
| Phe NH / Phe $C_\alpha$-H | 3.00 | 3.00 | 0.00 | 3.05 | 0.05 | 0.05 |
| Phe NH / Pro $C_\delta$-$H^{pro S}$ | 3.50 | 3.45 | – 0.05 | 3.50 | 0.00 | 0.05 |
| Phe NH / Ser $C_\beta$-$H^{pro R}$ | 3.50 | 3.50 | 0.00 | 3.15 | – 0.35 | – 0.35 |
| Ile $C_\alpha$-H / Ser NH | 2.50 | 2.60 | 0.10 | 3.00 | 0.50 | 0.40 |
| Ile $C_\delta$-H / Tyr $C_\delta$-H | 4.00 | 4.00 | 0.00 | 5.80 | 1.80 | 1.80 |
| Ile NH / Ile $C_\alpha$-H | 3.00 | 3.00 | 0.00 | 3.00 | 0.00 | 0.00 |
| Ile NH / Ile γ-Me | 3.00 | 3.75 | 0.75 | 5.10 | 2.10 | 1.35 |
| Pro $C_\delta$-$H^{pro S}$ / Ser $C_\alpha$-H | 2.30 | 2.20 | – 0.10 | 2.20 | – 0.10 | 0.00 |
| Pro $C_\delta$-$H^{pro S}$ / Ser $C_\beta$-$H^{pro R}$ | 2.50 | 2.40 | – 0.10 | 2.25 | – 0.25 | – 0.15 |
| Pro $C_\alpha$-H / Phe NH | 2.50 | 3.40 | 0.90 | 3.20 | 0.70 | – 0.20 |
| Gln $C_\alpha$-H / Ile NH | 2.50 | 2.40 | – 0.10 | 2.15 | – 0.35 | – 0.25 |
| Gln NH / Gln $C_\alpha$-H | 2.50 | 2.50 | 0.00 | 2.40 | – 0.10 | – 0.10 |
| Gln NH / Tyr $C_\alpha$-H | 3.00 | 3.75 | 0.75 | 3.75 | 0.75 | 0.00 |
| Ser $C_\beta$-H / Phe NH | 3.50 | 3.60 | 0.10 | 3.00 | – 0.50 | – 0.60 |
| Ser $C_\beta$-H / Tyr $C_{2,6}$-H | 4.00 | 4.00 | 0.00 | 4.25 | 0.25 | 0.25 |
| Ser NH / Ser $C_\alpha$-H | 3.00 | 3.00 | 0.00 | 3.00 | 0.00 | 0.00 |
| Ser NH / Ser $C_\beta$-$H^{pro S}$ | 2.50 | 2.60 | 0.10 | 2.45 | – 0.05 | – 0.15 |
| Tyr $C_\alpha$-H / Phe $C_{2,6}$-H | 4.00 | 4.60 | 0.60 | 5.75 | 1.75 | 1.15 |
| Tyr $C_\alpha$-H / Gln NH | 2.50 | 2.50 | 0.00 | 2.25 | – 0.25 | – 0.25 |

**Table S7.** NOE restraints and acquired distances for ncpA2. Please see Methods section 7 for explanations.

| NOE | target dist. | MD | Diff. | EM | Diff. | Diff. EM–MD |
|---|---|---|---|---|---|---|
| Phe $C_{2,6}$-H / Pro $C_\beta$-$H^{proR}$ | 3.00 | 3.15 | 0.15 | 5.55 | 2.55 | 2.40 |
| Phe $C_{2,6}$-H / Pro $C_\delta$-$H^{proS}$ | 3.00 | 2.95 | – 0.05 | 3.95 | 0.95 | 1.00 |
| Phe NH / Gln NH | 3.50 | 3.55 | 0.05 | 3.95 | 0.45 | 0.40 |
| Phe NH / Phe $C_\alpha$-H | 3.00 | 3.00 | 0.00 | 3.05 | 0.05 | 0.05 |
| Phe NH / Pro $C_\delta$-$H^{proS}$ | 3.50 | 3.45 | – 0.05 | 3.40 | – 0.10 | – 0.05 |
| Phe NH / Ser $C_\beta$-H | 3.50 | 3.45 | – 0.05 | 3.80 | 0.30 | 0.35 |
| Gln NH / Ser $C_\beta$-H | 3.50 | 3.70 | 0.20 | 4.00 | 0.50 | 0.30 |
| Ile $C_\alpha$-H / Ser NH | 3.00 | 3.05 | 0.05 | 3.00 | 0.00 | – 0.05 |
| Pro $C_\delta$-$H^{proR}$ / Ser $C_\alpha$-H | 2.30 | 2.25 | – 0.05 | 2.30 | 0.00 | 0.05 |
| Gln NH / Gln $C_\alpha$-H | 2.50 | 2.65 | 0.15 | 2.75 | 0.25 | 0.10 |
| Ser $C_\beta$-H / Phe NH | 3.50 | 3.45 | – 0.05 | 3.80 | 0.30 | 0.35 |
| Ser NH / Ser $C_\alpha$-H | 3.00 | 3.05 | 0.05 | 3.05 | 0.05 | 0.00 |
| Ser NH / Ser $C_\beta$-H | 2.50 | 2.50 | 0.00 | 2.60 | 0.10 | 0.10 |
| Phe $C_\alpha$-H / Phe CH=N | 2.30 | 2.45 | 0.15 | 2.80 | 0.50 | 0.35 |
| Phe CH=N / Tyr $C_\alpha$-H | 2.50 | 2.30 | – 0.20 | 2.65 | 0.15 | 0.35 |

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