Tesi Doctoral

NMR IN DRUG DISCOVERY. FROM SCREENING TO STRUCTURE-BASED DESIGN OF ANTITUMORAL AGENTS

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4 CONCLUSIONS

In view of the experiments presented throughout the previous chapters the conclusions of the present PhD thesis are the following:

4.1.1 CHAPTER 1

- We have successfully developed two novel labeling methodologies for the selective NMR observation of Trp, a residue frequently encountered on protein hot spots. First, we have benefited from the extensive knowledge on amino acid biosynthesis in bacteria to isotopically enrich with ^{13}C several positions on Trp's indole ring; namely C_2 and C_4. Later, we have been able to efficiently incorporate non-natural fluorinated Trp analogs into a protein thanks to a combined use of IAA, an inhibitor of tryptophan biosynthesis, and the non-natural fluorinated amino acid. All selective labeling schemes have proved able to monitor binding events on our model complex BIR3-AVPI peptide and, to some extent, even superior to traditional ^{15}N uniform labeling strategies; for this reason we anticipate interesting applications in the HTS field and also in the study of high molecular weight complexes.
- We have used WaterLOGSY experiments to screen a fragment library –composed by scaffolds frequently encountered in drugs - as a way to identify weak binders for Bcl-XL. Two novel compounds have been identified from such screening, one of which binds non-competitively to the protein with respect to BH3I-1 -previously reported by Degterev and collaborators. ILOE and competition STD experiments reveal that the latter ligands target adjacent sites on Bcl-XL binding groove. Later, using the interligand nOe contacts their relative orientation has been established and used to propose novel dual ligands.

4.1.2 CHAPTER 2

- We have successfully over-expressed, purified and refolded $VEGF_{11-109}$ as previously reported by Fairbrother and collaborators. Various protein batches, with different labeling schemes, have been produced to suit our experiment needs: uniform ^{15}N VEGF, selectively labeled $^{13}C_1$-Met VEGF and uniform ^{15}N-^{2}H-VEGF. Uniform ^{15}N labeling was achieved using an *E. coli* host and standard methods, while selective incorporation of ^{13}C in methionine side chains was carried out using an auxotrophic strain and the appropriately labeled amino acid. As for the perdeuterated protein batch, D_2O media and perdeuterated glucose were used to achieve a 97% substitution of all non-exchangeable protons by deuterons, as determined by MS spectrometry.
- A fragment-based drug discovery approach was used to devise all-D dipeptide libraries to be assayed during early NMR screenings. Reductionist strategies were applied to limit the number of synthesized dipeptides, first chemical diversity and later structure-based criteria to pick two different dipeptide subsets. Both libraries have been assayed using ligand-based NMR experiments -STD and WaterLOGSY- without our detecting any remarkable binder; which overall suggests that, despite their modularity and chemical accessibility, short oligopeptides may not be suited for fragment based drug discovery.
- A series of ^{15}N-^{1}H HSQC experiments have revealed that $VEGF_{11-109}$ is prone to bind small organic molecules, such as the organic solvents typically used in the preparation of chemical libraries. Titrations with various solvents (ACN, DMSO, iPrOH, dioxane and DMF) have been carried out and

in all cases residual binding occurs on the biologically relevant VEGF receptor binding epitope, although the effects differ both in extent and location for each solvent. Perturbation analyses for the various solvents allowed us to pin down relevant VEGF hot spots; in particular three different sub sites have been identified centered on M18, I46 and Q89, which fairly correlate with published mutagenesis studies. Overall, we conclude this approach could be applied as a general method to anticipate relevant hot spots and binding sites, as well as to assess the druggability of a given protein.

- Methyl-^{13}C-Met selectively labeled VEGF combined with the ^{13}C-filtered-^{13}C-decoupled ^{1}H NMR experiment were used to set up a very simple and cost efficient screening experiment. The lack of signal overlap coupled with the felicitous location of methionine residues on VEGF's surface allow us to monitor binding events with a very limited amount of protein and time; while at the same time being able to assay complex compound mixtures and preserving structural insight into their eventual binding. This scheme was successfully applied to screen and identify two positive mixtures out of a library of water extracts derived from plants employed in traditional Chinese medicine.
- The main component in *Radix scutellariae* water extract was isolated and identified as the active compound against VEGF. Both MS and NMR spectroscopic data indicate that the isolated molecule is a flavonoid compound: baicalin. In fact, exploration of several other family members leads to the identification of quercetin-3-β-glucoside, a slightly tighter ligand. Several titrations provided us with their dissociation constant values; while differential analysis of baicalin and quercetin-3-β-glucoside's perturbation profiles on ^{15}N-^{1}H HSQC experiments together with STD experiments let us propose a model for the binding of these molecules. According to this, flavonoid binding competes with v107 peptide for VEGF dimer interface sitting surrounded by the 2nd β-strand, N-terminus helix and the loop connecting 3rd and 4th β strands.
- Two green tea catechins were also assayed for their VEGF binding properties. They belong to the flavonoid compound family and several authors have report antiangiogenic activity, possibly through VEGF signaling pathway inhibition. Our results support such mechanism, especially for EGCG, which shares similar affinity and ^{15}N-^{1}H HSQC perturbation profiles with quercetin-3-β-glucoside, although a considerable propensity to hydrolysis of its gallate moiety was observed under our sample conditions.
- Finally, we considered the use of Cross-saturation TROSY experiment to accurately map VEGF binding sites with very weak ligands. The experiment was successfully implemented and several of its parameters explored. However, the results for VEGF complex with v107 peptide and some other weaker binders suggest a limited applicability of the experiment; which mainly stems from incomplete deuteration and subsequent residual saturation on VEGF.

4.1.3 CHAPTER 3

- Characterization of Kahalalide F conformational propensities in water and DMSO has been carried out using NMR spectroscopy with similar results in both conditions. The peptide is mainly unstructured at its N-terminus region as revealed by the lack of non-sequential nOes. On the

contrary, the C-terminus macro cycle displays a stiffer nature and presents two tight turns stabilized by the same trans-annular hydrogen bond pattern. Furthermore, under water conditions various observations denote certain peptide amphipathicity, along with a tendency to organize into supramolecular assemblies.

- We have also explored Kahalalide F conformational propensities in membrane mimicking environments, namely in detergent micelles. We have carried out a variety of NMR experiments on peptide samples with the presence of SDS micelles and eventually calculated its structure using a simulated annealing protocol. Above SDS's critical micellar concentration Kahalalide F inserts into the detergent aggregate to form a mixed micelle; under such conditions, the N-terminus portion of the peptide adopts a tight β-II turn centered around Pro 6 so that Orn 7 side chain remains close to the detergent polar heads and the hydrophobic methyl hexanoic acyl moiety sits in the micelle's core. As for Kahalalide F C-terminus, the same hydrogen bond pattern is established as a result of the $\alpha-\beta$ dehydroamino acid induced β-turn and an unconventional turn around the depsipeptidic linkage, yielding a rigid and rather flat macro cycle that is partly embedded within the detergent micelle.
- A model for the monomeric insertion of Kahalalide F within membrane-like environments has been presented and supports that part of the peptide's cytotoxic activity occurs at a membrane level; for which the turn within its N-terminus section seems important. This model is however not complete, as a mode of action involving membrane destabilizing effects of peptide supramolecular assemblies is very plausible given the peptide propensity to aggregation in water.

5 MATERIALS AND METHODS

5.1 NMR METHODS IN DRUG DISCOVERY: A HANDS ON EXPERIENCE

5.1.1 PROTEIN PRODUCTION

5.1.1.1 ^{15}N LABELED XIAP-BIR3

Recombinant BIR3 was over expressed as a His-tag fusion protein using the BL21 *E. coli* strain and M9 minimal media. Cells where grown at 37 °C in 2 L flasks until (OD_{600}) 0.8; at this point protein expression was induced for 4h at 37°C by adding 1 mM IPTG. ^{15}N uniform labeling was achieved by using $^{15}NH_4Cl$ (1g/L) as nitrogen source. Following cell lysis, soluble protein was purified over a His-trap chelating column (Amersham, Pharmacia) and thoroughly dialyzed into sample buffer. Reverse labeling on Trp side chain nitrogen was achieved with the addition of ^{14}N indole (Cambridge Isotopes) prior to IPTG induction.

5.1.1.2 SELECTIVELY LABELED XIAP-BIR3

Trp selectively labeled BIR3 was over expressed similar to 5.1.1.1. Cells were grown at 37 °C in 2 L shaker flasks until (OD_{600}) 0.8. Then, 50 mg of [2-^{13}C] or [4-^{13}C] labeled indole (Cambridge Isotopes) was added to the media as a suspension in 1 mL of DMSO prior IPTG induction (4 h at 37 °C). Following cell lysis, soluble protein was purified over a His-trap chelating column (Amersham, Pharmacia) and thoroughly dialyzed into sample buffer.

5.1.1.3 INHIBITION OF TRP BIOSYNTHESIS BY 3 -INDOLEACRILIC ACID

The inhibitory effect of IAA on cell growth was tested by growing E. coli BL21 strain cells in M9 minimal media in the presence of IAA (SIGMA, 50 mg/L $_1$) and with or without L-Trp (SIGMA, 50 mg/L $_1$). IAA and $_L$ Trp were added to the media dispersed in dimethylsulfoxide (1 mL) with stirring. The cells were grown at 37 ºC in 2-L shaker flasks. To monitor cell growth, the optical density at 600 nm (OD_{600}) was measured every 30 minutes for seven hours. As a further control, we also verified that IAA growth inhibition could be reversed by L-Trp. IAA-treated cells (no growth after 7 h) were grown overnight after addition of L-Trp (50 mg/L 1) and reached a final OD_{600} of 1.6. The experiments were repeated twice under the same conditions to confirm the reproducibility of our data.

5.1.1.4 F-TRP XIAP-BIR3

Recombinant BIR3 was expressed as a His-tag protein in BL21 strain E. coli cells and M9 minimal media. Cells were grown at 37 ºC in 2-L shaker flasks until OD_{600} 1. At this point, two different expression procedures were carried out. Just before induction, 5F-Trp (SIGMA, 50 mg/L) and IAA (SIGMA, 10 mg/L) were added to one aliquot medium (1 L), while only 5F-Trp (SIGMA, 50 mg/L) was added to another aliquot of medium (1 L). Induction (4 h, 37 ºC) was achieved with IPTG (1 mM). Following cell lysis, His-tagged soluble protein was purified over a Hi-trap chelating column (Amersham, Pharmacia) and dialyzed into sample buffer.

5.1.2 CHEMICAL LIBRARIES

5.1.2.1 FRAGMENT LIBRARY

Reagents were purchased from various sources: Chembridge, Sigma, Acros, etc. Compounds were weighted and dissolved in DMSO-d_6 at a 100 mM concentration, later their chemical identity, integrity and water solubility were checked performing 1H NMR experiments in buffered water conditions.

5.1.3 NMR SPECTROSCOPY

5.1.3.1 SPECTROMETERS

Varian Unity+ 500 MHz (The Burnham Institute, CA). The spectrometer was equipped with a triple resonance 5mm pentaprobe with gradients on the Z axis.

Bruker Avance 600MHz (Scripps Institute, CA) The spectrometer was equipped with a 5 mm $^{19}F/^{1}H$ probe.

5.1.3.2 BIR3 EXPERIMENTS

^{15}N-^{1}H HSQC

^{15}N-^{1}H HSQC spectra were recorded for samples containing 0.7 mM BIR3 and 20 mM phosphate buffer at pH 7.5. 160x1024 complex points were acquired at 303 K with 32 transients per increment leading to a total measurement time of 1h and 30 minutes per spectrum.

^{13}C-^{1}H HMQC

Spectra were recorded with 0.2 mM BIR3 sample in 20 mM phosphate D_2O buffer at pH 7.5 and 30 °C. These were measured on Varian Unity+ operating at a 500 MHz ^{1}H frequency. 64x1024 complex points were acquired with 64 transients per increment leading to a total measurement time of 1 h 30 min per spectrum.

1D ^{13}C filtered ^{13}C decoupled ^{1}H NMR spectra

Spectra were measured with 96 transients on a Varian Unity+ (total measurement time was rougly 3 min). Sample consisted on a 200 μM protein sample in 20 mM phosphate D_2O:H_2O (9:1) buffer at pH 7.5.

^{19}F NMR spectroscopy

^{19}F NMR spectra were acquired on a Bruker 600Mhz; collected at 25 °C with 0.1mM samples of ^{19}F-Trp labeled BIR3 in H_20/D_20 (90:10) phosphate buffer (40 mM, pH 7.5). Spectral width was 11.26 MHz; 16 K data points and 256 scans were collected for a total measurement time of approximately 10 minutes per experiment. For processing, we used the software MestreC, and a 10 Hz line-broadening weighting function was applied. Internal trifluoroacetic acid (0.0 ppm) was used as the reference.

5.1.3.3 BCL-XL EXPERIMENTS

^{15}N-^{1}H HSQC

^{15}N-^{1}H HSQC spectra were recorded for samples containing 0.25 mM Bcl-XL and 20 mM phosphate buffer at pH 7.5. 160x1024 complex points were acquired at 298 K with 32 transients per increment leading to a total measurement time of 1h and 30 minutes per spectrum

WaterLOGSY and Saturation Transfer Difference

Both experiments were performed using the same pulse sequence on a 500 MHz Varian Unity spectrometer. For WaterLOGSY experiment on-resonance irradiation was performed on water signal with a train of selective IBURP2 pulses of 7 ms durations, spaced by a 10 ms delay. Total saturation time used was 2.5s and water suppression was achieved with a WATERGATE module. On-resonance irradiation for saturation transfer difference experiment is performed on the aliphatic protein region. Also in both experiments on and off-resonance experiment subtraction can either be carried out interleaved or sequentially.

Interligand nOe and trNOESY

ILIOE experiments were measured on a 500 MHz Varian Unity spectrometer, and its practical details are those of a typical NOESY spectrum. Mixing times are usually around 300-600 ms to maximize the detection of trNOEs and ILOEs.

In the case of either ILOE or WaterLOGSY experiments, sample consists in mixtures of compounds (0.5 mM to 1 mM each) and 10μM Bcl-XL in a 20 mM phosphate buffer at pH 7.5.

5.2 VEGF ANTAGONIST DESIGN

5.2.1 PROTEIN PRODUCTION

5.2.1.1 ^{15}N UNIFORMLY LABELED VEGF

E. coli cells were transformed with p6XHisVEGF$_{11-109}$ and pMS421 plasmids and plated on solid LB/Agar media. 5ml of overnight carbenicillin/spectinomycin containing LB rich media were inoculated into 500 ml M9 minimal media containing 1 g/L $^{15}NH_4Cl$ as nitrogen source. Growth was monitored using optical density at 600nm until this reached 0.8, to be induced with 1 mM IPTG for 5 hours at 37ºC.

Harvested cells were dissolved in 6 M guanidine HCl, 0.1 M NaH_2P0_4, 10 mM Tris at 10 mM 2-mercaptoethanol pH 8 and the filtered lysate applied to a Ni affinity column under denaturing purification conditions; the protein is eluted with 8 M urea, 0.1 M NaH_2P0_4, 20 mM Tris HCl, 0.5 M imidazole, pH 5.9. Fractions are analyzed by SDS-PAGE and VEGF containing fractions are selected and pooled together. Protein concentration was estimated both with Bradford assay and A_{280} and diluted to 1mg/ml concentration. DTT was added at 20mM and reduction proceeded for 3 hours with gentle stirring.

Refolding proceeds by stepwise removal through dialysis (MWCO 6000-8000 Da) of denaturing and reducing agents from the initial solution (8M urea, 20mM cysteine) at 4°C, the final buffer being 20 mM Tris HCl buffer at pH 8.2. Refolding yield is assed by non-reducing SDS-PAGE.

His-tag proteolytic cleavage is performed using 100:1 (VEGF:Genenase I) ratio in 20mM Tris HCl buffer at pH 8.2, 200 mM NaCl and 1mM EDTA overnight at room temperature. Digested protein is dialyzed into 20mM Tris HCl pH 7.5 at 4°C. An anion exchange purification step is carried out with HP-Q sepharose column using a 20mM Tris HCl pH 7.5 buffer at 4°C and eluted with NaCl gradient up to 1mM. Protein eluates are analyzed by non-reducing SDS-PAGE and VEGF containing fractions pooled together and concentrated by ultrafiltration prior to S75 size exclusion purification.

S75 size exclusion is performed using 25mM phosphate buffer pH 7, 50mM NaCl. Pure protein fractions are checked by non-reducing SDS-PAGE; finally protein is quantified using Bradford assay, concentrated by ultrafiltration to 10 mg/ml and flash-frozen for long-term storage.

5.2.1.2 SELECTIVELY LABELED METHYL-^{13}C-MET VEGF

Selectively labeled methyl-^{13}C-Met VEGF is produced as the ^{15}N uniformly labeled counterpart, except that methionine auxotrophic E. coli strain (B843) is used instead. Also M9 minimal media is supplemented with methyl-13C-Met (50 mg/L).

5.2.1.3 ^{15}N-^{2}H UNIFORMLY LABELED VEGF

Perdeuterated ^{15}N uniformly labeled VEGF is produced as in 5.2.1.1 except that M9 media is prepared using D_2O, Glucose-d_{12} (2 g/L) and $^{15}NH_4Cl$ (1 g/L). Also E. coli were acclimatized to D_2O in a stepwise fashion: initially, picked cells are inoculated into of 50 mL 50% D_2O media and grown overnight; subsequently 250 ml of 100% D2O media are inoculated with all previous cells and grown to OD 0.6; at this point cells are collected, resuspended, and inoculated into 1L 100% D_2O minimal media.

5.2.2 CHEMICAL LIBRARIES

5.2.2.1 DIPEPTIDE LIBRARIES

Dipeptide libraries, were supplied by Francesc Yraola at Combinatorial Chemistry Unit in the PCB. Their synthesis was carried out on Solid Phase Synthesis and purified by HPLC to a purity 95% or higher. For storage purposes compounds were weighted and dissolved in DMSO-d_6 at a 100 mM concentration, later their chemical identity, integrity and water solubility were checked performing 1H NMR experiments in buffered water conditions (0).

5.2.2.2 TRADITIONAL CHINESE MEDICINE PLANT EXTRACTS

TCM plants were obtained from Herbasin (Shenyang, China). Dried plant material (30 g) was extracted with H_2O (400 mL) at reflux for 5h by use of a Soxhlet apparatus. The volume of the aqueous extracts was reduced by evaporation under vacuum and the extract was afterwards freeze-dried. Finally, DMSO-d_6 concentrated stocks were prepared at a 200 mg/ml concentration.

5.2.3 SAMPLE PREPARATION

STD and WaterLOGSY

Ligand detected experiments were recorded with 10 μM VEGF and 500 μL samples. This was obtained by diluting concentrated protein stock (10 mg/mL) in buffered D_2O (pH 7, 25 mM phosphate, 50 mM NaCl solution and 9:1 D_2O:H_2O). Assayed compound were diluted from their DMSO stocks to 0.5 or 1 mM concentrations.

^{13}C-decoupled-^{13}C-filtered 1H NMR

CSP assays using methionine selectively labeled VEGF were performed on 30 μM protein samples; buffer was pH 7 25 mM phosphate, 50 mM NaCl solution in 95% D_2O.

^{15}N, ^{13}C-^{1}H HSQC

Protein 2D heteronuclear experiments were acquired on 250 μM *methyl-^{13}C*-Met VEGF in 50 mM NaCl, pH 7 25mM phosphate buffer using Shigemi tubs.

^{15}N-^{2}H Cross Saturation TROSY

Cross-saturation TROSY-HSQC experiments were performed on for VEGF and VEGF-v107 complex. In both samples protein concentration was 250μM ^{15}N-^{2}H VEGF, and when v107 peptide was added to the sample this was done at a 1mM concentration. Buffer at pH 7 was composed of 50 mM NaCl and 25 mM phosphate in 9:1 H_2O:D_2O.

5.2.4 NMR SPECTROSCOPY

5.2.4.1 SPECTROMETERS

Bruker Digital Avance 600MHz (Unitat de RMN d'Alt Camp de la Universitat de Barcelona, Parc Cientific Barcelona, Serveis Científico Tècnics). The spectrometer was equipped with a triple resonance (^{1}H, ^{13}C, ^{15}N) TXI 5mm probe with gradients on axis X,Y and Z.

Varian Inova 500 Mhz (Unitat de RMN d'Alt Camp de la Universitat de Barcelona, Parc Cientific Barcelona, Serveis Científico Tècnics). The spectrometer was equipped with a triple resonance 5mm pentaprobe with gradients on the Z axis.

5.2.4.2 EXPERIMENTS

WaterLOGSY and Saturation Transfer Difference

Both experiments were performed using the same pulse sequence either on a 500 MHz Varian Inova or Bruker 600MHz spectrometer. For WaterLOGSY experiment on-resonance irradiation was performed on water signal with a train of selective IBURP2 pulses of 7 ms or 25ms long Gaussian pulses, spaced by a 10 ms delay. In any case total saturation time used was 2.5s and water suppression was achieved with a WATERGATE module. On-resonance irradiation for saturation transfer difference experiment is performed on the aliphatic protein region. Also in both experiments on and off-resonance experiment subtraction can either be carried out interleaved or sequentially.

^{13}C-decoupled-^{13}C-filtered ^{1}H and ^{13}C-^{1}H HSQC NMR

^{13}C-^{1}H HSQC spectra were recorded on Bruker 600 with a 3000Hz spectral width on ^{13}C, and 40x2048 complex points were acquired with a total of 32 transients per increment.

^{13}C-decoupled-^{13}C-filtered ^{1}H experiments on the other hand, were recorded on the Bruker 600 Mhz spectrometer with a 9000Hz ^{1}H spectral width, 2048 points and a total of 128 transients.

^{15}N-^{1}H HSQC

^{15}N-^{1}H HSQC spectra were recorded both on Bruker 600 and Varian 500 spectrometers. 128x1024 complex points were acquired with a total of 32 transients per increment.

Cross-saturation TROSY-^{15}N-^{1}H HSQC

Cross saturation experiment was implemented on the Varian Unity spectrometer with the on and off-resonance experiments acquired in an interleaved fashion. Saturation module was set up using c.w irradiation at 6dB, or Gaussian shaped pulses with different power and lengths for 2.5s. Different recycling (1-3s) delays and on resonance irradiation frequencies (0-3ppm) were explored.

5.3 KAHALALIDE F STRUCTURAL CHARACTERIZATION

5.3.1 NMR SPECTROSCOPY

5.3.1.1 SPECTROMETERS

Bruker Digital Avance 600MHz (Unitat de RMN d'Alt Camp de la Universitat de Barcelona, Parc Cientific Barcelona, Serveis Científico Tècnics). The spectrometer was equipped with a triple resonance (^{1}H, ^{13}C, ^{15}N) TXI 5mm probe with gradients on axis X,Y and Z.

Bruker Digital Avance 800MHz (Unitat de RMN d'Alt Camp de la Universitat de Barcelona, Parc Cientific Barcelona, Serveis Científico Tècnics). The spectrometer was equipped with a triple resonance (^{1}H, ^{13}C, ^{15}N) TXI 5mm probe with gradients on axis X,Y and Z.

5.3.1.2 EXPERIMENTS

Temperature coefficients

Temperature coefficients were obtained on the Bruker 600 spectrometer under all conditions using a series of 3-9-19 watergate ^{1}H experiments at different temperatures: 283, 288, 293, 298, 303, 308, and 313 K, using 4K acquisition points, 64 transients and 2 s recycling delay.

Deuterium Exchange

^{1}H Watergate experiments evenly spaced 5min in time were acquired on the Bruker 600. These were recorded at 283 K with a 3-9-19 water suppression scheme, 2 s recycling delay and 4K points. Following acquisition, each experiment was processed, the amide peaks assigned and its intensity measured.

TOCSY

TOCSY experiments used, either in the assignment process or in the experiments with paramagnetic reagents, were performed with an 80 ms mixing time and 256x2048 complex points.

NOESY

NOESY experiments used during the assignment and structural calculation were acquired on Bruker 600 and 800 spectrometers. A variety of mixing times (200-600 ms) and transient numbers were used obtain a total 512x2048 complex points.

5.3.2 XPLOR SIMULATED ANNEALING

5.3.2.1 PROTOCOL

Structural calculations for Kahalalide F in SDS/water media were performed using a modified version of the standard simulated annealing protocol (sa.inp) available in Xplor. Starting annealing temperature was set to 1000 K, the high-temperature stage lasted for 24200 steps; while 6000 steps were performed during the cooling stage. The initial template structure was generated using standard protocol (*generate_template.inp*). Finally, in order to allow for a better sampling of the conformational space Val14- AlloThr9 ester bond energy is scaled down during high-energy stages, and later rescaled during the cooling phase.

```
remarks file  nmr/sa.inp
remarks  Simulated annealing protocol for NMR structure determination.
remarks  The starting structure for this protocol can be any structure with
remarks  a reasonable geometry, such as randomly assigned torsion angles or
remarks  extended strands.
remarks  Author: Michael Nilges
remarks  Modification to decrease depsipeptidic link strenght during initial
remarks  stages of SA protocol.
remarks  Ricard 15.11.04
{====>}
evaluate ($init_t = 1000 )      {*Initial simulated annealing temperature.*}
{====>}
evaluate ($high_steps= 24200 )        {*Total number of steps at high temp.* 24000}
{====>}
evaluate ($cool_steps = 6000 )     {*Total number of steps during cooling.* 3000}
parameter                           {*Read the parameter file.*}
{====>}
   @/Users/Ricard/DATA/KHF/KHF_struct/CALC/toppar/charmm19/parallhdg.pro
   @/Users/Ricard/DATA/KHF/KHF_struct/CALC/toppar/charmm19/KF_rar.par
end
{====>}
structure @../KF.psf end                    {*Read the structure file.*}
{====>}
coordinates @../KF_temp.pdb                 {*Read the coordinates.*}
noe
{====>}
   nres=3000           {*Estimate greater than the actual number of NOEs.*}
   class all
{====>}
 {*Read NOE distance ranges.*}
  @/Users/ricard/DATA/KHF/KHF_struct/CALC/charmm19/frag_KF/cycle/SA/kf_noe200_NH_manual.tbl
  @/Users/ricard/DATA/KHF/KHF_struct/CALC/charmm19/frag_KF/cycle/SA/kf_noe200_Ca_manual.tbl
  @/Users/ricard/DATA/KHF/KHF_struct/CALC/charmm19/frag_KF/tail/SA/kf_noe200_tail_NH_manual.tbl
  @/Users/ricard/DATA/KHF/KHF_struct/CALC/charmm19/frag_KF/tail/SA/kf_noe200_tail_Ca_manual.tbl
  @kf_noe200_link_Ca_manual.tbl
  @kf_noe200_link_NH_manual.tbl
 { hydrogen bonds}
{  @/Users/ricard/DATA/KHF/KHF_struct/CALC/charmm19/frag_KF/cycle/SA/kf_hbond_cycle.tbl }
end
{====>}
{restraints dihedral
   nass = 1000
   @dihe.tbl                    {*Read dihedral angle restraints.*}
end}
{* Reduce the scaling factor on the force applied to depsipeptidic linkage           *}
{* bonds and angles from 1000.0 to 100.0 in order to reduce computation instability. *}
parameter
     bonds ( name OG1 ) ( name C ) 100. TOKEN
     angle ( name CA ) ( name C ) ( name OG ) 50. TOKEN
     angle ( name C ) ( name OG1 ) ( name CB ) 50. TOKEN
end
flags exclude * include bonds angle impr vdw noe cdih end
                    {*Friction coefficient for MD heatbath, in 1/ps.   *}
vector do (fbeta=10) (all)
                    {*Uniform heavy masses to speed molecular dynamics.*}
vector do (mass=100) (all)
noe                         {*Parameters for NOE effective energy term.*}
  ceiling=1000
  averaging  * cent
  potential  * square    {only upper bounds, nOe file modified accordingly, Ricard}
  scale      * 50
  sqoffset   * 0.0
  sqconstant * 1.0
  sqexponent * 2
  soexponent * 1
  asymptote  * 0.1                      {*Initial value--modified later.*}
  rswitch    * 0.5
end
```

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parameter                    {*Parameters for the repulsive energy term.*}
   nbonds
     repel=1.               {*Initial value for repel--modified later.*}
     rexp=2 irexp=2 rcon=1.
     nbxmod=3
     wmin=0.01
     cutnb=4.5 ctonnb=2.99 ctofnb=3.
     tolerance=0.5
  end
end
restraints dihedral
     scale=5.
end
{====>}
evaluate ($end_count=50)         {*Loop through a family of 10 structures.*}
coor copy end
evaluate ($count = 0)
while ($count < $end_count ) loop main
  evaluate ($count=$count+1)
  coor swap end
  coor copy end
   {* ============================================== Initial minimization.*}
  restraints dihedral   scale=5.   end
  noe asymptote * 0.1  end
  parameter  nbonds repel=1.   end end
  constraints interaction
        (all) (all) weights * 1  vdw 0.002 end end
  minimize powell nstep=50 drop=10.  nprint=25 end
   {* ======================================== High-temperature dynamics.*}
  constraints interaction (all) (all)
           weights * 1  angl 0.4  impr 0.1 vdw 0.002 end end
  evaluate ($nstep1=int($high_steps * 2. / 3. ) )
  evaluate ($nstep2=int($high_steps * 1. / 3. ) )
  dynamics  verlet
    nstep=$nstep1   timestep=0.005   iasvel=maxwell   firstt=$init_t
    tcoupling=true  tbath=$init_t  nprint=50  iprfrq=0
  end
   {* ============== Tilt the asymptote and increase weights on geometry.*}
  noe asymptote * 1.0  end
  constraints interaction
          (all) (all) weights * 1  vdw 0.002  end end
  {* Bring scaling factor for depsipeptidic bonds back *}
  parameter
    bonds ( name OG1 ) ( name C ) 1000. TOKEN
    angle ( name CA ) ( name C ) ( name OG ) 500. TOKEN
    angle ( name C ) ( name OG1 ) ( name CB ) 500. TOKEN
  end
  dynamics  verlet
    nstep=$nstep2   timestep=0.005 { timestep=0.005 }    iasvel=current   tcoupling=true
    tbath=$init_t  nprint=50  iprfrq=0
  end
  {* ================================================  Cool the system.*}
  restraints dihedral   scale=200.   end
  evaluate ($final_t = 100)      { K }
  evaluate ($tempstep = 50)      { K }
  evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
  evaluate ($nstep = int($cool_steps/$ncycle))
  evaluate ($ini_rad  = 0.9)        evaluate ($fin_rad  = 0.75)
  evaluate ($ini_con=  0.003)       evaluate ($fin_con=  4.0)
  evaluate ($bath  = $init_t)
  evaluate ($k_vdw = $ini_con)
  evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
  evaluate ($radius=    $ini_rad)
  evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))
  evaluate ($i_cool = 0)
  while ($i_cool < $ncycle) loop cool
    evaluate ($i_cool=$i_cool+1)
    evaluate ($bath  = $bath  - $tempstep)
    evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
    evaluate ($radius=max($fin_rad,$radius*$radfact))
```

```
    parameter  nbonds repel=$radius   end end
    constraints interaction (all) (all)
                weights * 1. vdw $k_vdw end end
    dynamics  verlet
      nstep=$nstep  time=0.005 iasvel=current firstt=$bath
      tcoup=true tbath=$bath nprint=$nstep iprfrq=0
    end
{====>}                                        {*Abort condition.*}
    evaluate ($critical=$temp/$bath)
    if ($critical >  10. ) then
      display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
      stop
    end if
  end loop cool
  {* ================================================= Final minimization.*}
  constraints interaction (all) (all) weights * 1. vdw 1. end end
  parameter
    nbonds
      repel=0.8 {original value 0.8}
      rexp=2 irexp=2 rcon=1.
      nbxmod=3
      wmin=0.01
      cutnb=6.0 ctonnb=2.99 ctofnb=3.
      tolerance=1.5
    end
  end
  minimize powell nstep=1000 drop=10.0 nprint=25 end { nstep changed from 1000 to 2000}
  {* ================================== Write out the final structure(s).*}
  print threshold=0.5 noe
  evaluate ($rms_noe=$result)
  evaluate ($violations_noe=$violations)
  print threshold=5. cdih
  evaluate ($rms_cdih=$result)
  evaluate ($violations_cdih=$violations)
  print thres=0.05 bonds
  evaluate ($rms_bonds=$result)
  print thres=5. angles
  evaluate ($rms_angles=$result)
  print thres=5. impropers
  evaluate ($rms_impropers=$result)
  remarks ============================================================
  remarks           overall,bonds,angles,improper,vdw,noe,cdih
  remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
  remarks ============================================================
  remarks           bonds,angles,impropers,noe,cdih
  remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
  remarks ============================================================
  remarks              noe,  cdih
  remarks violations.: $violations_noe, $violations_cdih
  remarks ============================================================
{====>}                     {*Name(s) of the family of final structures.*}
  evaluate ($filename="sa_"+encode($count)+".pdb")
  write coordinates output = $filename  end
end loop main
stop
```

5.3.2.2 FORCE FIELD

The force field parameters used in the previous simulated annealing protocol correspond to a modified version of Xplor force field applied to calculations using NMR restrains. Xplor's force field resembles very closely Charmm19, in our case several modifications have been introduced in order to account for Kahalalide F peculiarities: ester bond and dehydroamino acid olefin; for which geometric and energetic parameters have been added.

```
REMARK   addendum to file parallhdg_*.pro
REMARK   to be used for KF-like molecules
REMARK   Modified by  Ricard Rodriguez Feb/04

set echo=false  end
! BONDS
 BOND  OS   CT      $kbon {340.000} {SD=     .030}     1.430 ! ALLOW POL PEP     ! from charmm22     OS   CT3
 BOND  OS   C       $kbon {150.000} {SD=     .044}     1.334 ! ALLOW POL PEP     ! from charmm22     OS   CD  ! BONDS involving  CUA1 for
DhB residue from CHARMm.parm XPLOR/InsightII
 BOND CT   CUA1   $kbon  {300.0}      1.51
 BOND CUA1 CUA1   $kbon  {590.0}      1.343
 BOND CUA1 NH1    $kbon  {390.0}      1.47
 BOND HA   CUA1   $kbon  {365.0}      1.074
 BOND C    CUA1   $kbon  {282.0}      1.476
!-------------------------------------------------------------------------
! ANGLE
 ANGLE  CT  OS   C    $kang     {40.00} {SD=     .086}    109.6000    UB     30.000       2.265 ! ALLOW  POL PEP      ! from charmm22    CT2  OS
CD
 ANGLE  OS  CT   CT   $kang     {75.70} {SD=     .063}    110.1000 ! ALLOW   ALI ALC ARO     ! from charmm22    OH1  CT1  CT1
 ANGLE  OS   CT  HA   $kang     {60.00} {SD=     .070}    109.5000 ! ALLOW PEP POL     ! From charmm22    OS   CT2  HA
 ANGLE  OS   C    CT  $kang     {55.00} {SD=     .073}    109.0000    UB     20.000       2.326 ! ALLOW POL PEP    ! from charmm22    OS   CD  CT1
 ANGLE  OS   C    OC  $kang     {90.00} {SD=     .057}    125.9000    UB    160.000       2.258 ! ALLOW  PEP POL   ! from charmm22    OS   CD  OB
! ANGLE involving  CUA1 for DhB residue from CHARMm.parm XPLOR/InsightII
ANGLE CT   C    CUA1    $kang    {70.0}     117.5
ANGLE CUA1 C    NH1     $kang    {55.0}     119.0
ANGLE CUA1 C    O       $kang    {40.0}     120.0
ANGLE CUA1 CUA1 NH1     $kang    {50.0}     122.0
ANGLE C    CUA1 CUA1    $kang    {72.0}     119.5
ANGLE C    CUA1 NH1     $kang    {50.0}     118.0
ANGLE CT   CUA1 HA      $kang    {35.0}     120.0
ANGLE CT   CUA1 CUA1    $kang    {40.0}     122.9
ANGLE HA   CUA1 CUA1    $kang    {35.0}     119.4
ANGLE C    NH1  CUA1    $kang    {60.0}     120.0
ANGLE H    NH1  CUA1    $kang    {35.0}     120.0
ANGLE HA   CT   CUA1    $kang    {50.0}     110.00
!-------------------------------------------------------------------------
!DIHEDRALS
! The calculation doesn't crash eventhough the dihedrals below are missing
{ DIHEDRAL  OB   C   OS   CT    MULTIPLE= 2      $kdih {.96}     1    180.0000 ! ALLOW   PEP POL     ! based on OB   CD   OS   CT2
                                $kdih {3.85}     2    180.0000 ! ALLOW   PEP POL     ! based on OB   CD   OS   CT2 Redundant? }
 DIHEDRAL  X   C   OS   X        $kdih {2.05} {SD=      .380}    2    180.0000 ! ALLOW   PEP POL  ! based on X    CD   OS   X  from charmm22
 DIHEDRAL  X   CT  OS   X        $kdih {-.10} {SD=999999.000}    3      .0000 ! ALLOW   PEP POL  ! based on X    CT2   OS   X from charmm22

!Backbone Dihedrals for Dehydro AAs. sould be finetuned ( From MSI/Insight)
 DIHEDRAL X    CUA1 NH1  X      $kdih {0.48} 2  180.0  !modified according to Teochem 431, (1998) 79-96
 DIHEDRAL X    C    CUA1 X      $kdih {0.9}  2  180.0

! the way we constructed the molecule puts this dihedrals, nedded in refine_gentle.inp 06.05.05
! dihedrals are not used in the calculation $kdih=0 , if they were to be used we should look into this
! throughly.
 DIHEDRAL  X   N   NH1   X       $kdih {2.50} {SD=     .344}    2    .0000 !  ALLOW PEP
 DIHEDRAL  X   N   CT    X       $kdih {2.50} {SD=     .344}    2    .0000 !  ALLOW PEP
 DIHEDRAL  X   N   C   X       MULTIPLE=2    $kdih {2.50} {SD=     .344}    1   .0000 !
                                                              $kdih {2.50} {SD=     .344}    2    .0000 !  ALLOW PEP

 DIHEDRAL  CT   C   NH1   CT    MULTIPLE=2    $kdih {2.50} {SD=     .344}    1   .0000 !  ALLOW PEP
                                                                    $kdih {2.50} {SD=     .344}    2   .0000 !  ALLOW PEP
 DIHEDRAL  CT   C   NH1   H       $kdih {2.50} {SD=     .344}    2    .0000 !  ALLOW PEP
 DIHEDRAL  O   C   NH1   H     $kdih {2.50} {SD=     .344}    1   .0000 !  ALLOW PEP
 DIHEDRAL  O   C   NH1   CT    MULTIPLE=2    $kdih {2.50} {SD=     .344}    1   .0000 !  ALLOW PEP
                               $kdih {2.50} {SD=     .344}    2   .0000 !  ALLOW PEP
 DIHEDRAL  X   C   OS  X       $kdih {2.50} {SD=     .344}    2    .0000 !
 DIHEDRAL  X   C   NH1 X    MULTIPLE=2    $kdih {2.50} {SD=     .344}    1   .0000 !
                                      $kdih {2.50} {SD=     .344}    2    .0000 !
 DIHEDRAL  X   N   CP  X    MULTIPLE=2    $kdih {2.50} {SD=     .344}    1   .0000 !
                              $kdih {2.50} {SD=     .344}    2    .0000 !
!-------------------------------------------------------------------------
! IMPROPER
 IMPROPER   HA   CT  OS   CT     $kchi    0   65.977  ! Thr CB chirality (with DEPSI patch)
 IMPROPER   HA   OS  CT   CT     $kchi    0   65.977  ! allo-Thr CB chirality (with DEPSI patch)
```

```
 IMPROPER   CUA1 C   NH1  CT     $kpla    0   180    ! Trans peptide bonf Dehydro aa
 IMPROPER   CT   C   NH1  CUA1   $kpla    0   180    ! Trans peptide bonf Dehydro aa
! impropers involving CUA1 adapted from XPLOR/InsightII
IMPROPER CUA1 X    X    NH1    $kpla {45.0}  0    0.0 ! force planarity (Sp2 carbon)
IMPROPER HA   X    X    CUA1   $kpla {75.0}  0    0.0 ! force planarity (Sp2 carbon)
IMPROPER CT   X    X    CUA1   $kpla {70.0}  0    0.0 !> translated for our atom defin.
IMPROPER X    CUA1 CUA1 X      $kpla        0    0.0 ! included to hold CA=CB planarity
!improper HA   HA   CT   CUA1 $kchi 0 -70.874  !we don't need this so far (from msi files)
!---------------------------------------------------------------------------
! NONBONDED
 NONBONDED  OS      .1521   3.1538    .1521   3.1538 ! ALLOW   ALC ARO  ! from charmm22 OS
! NONBONDED involving  CUA1 for DhB residue from CHARMm.parm XPLOR/InsightII
NONBonded  CUA1  0.04000   3.4923   0.10000   3.1360
set echo=true  end
```

5.3.2.3 NOE RESTRAINTS

nOe restraints for Kahalalido F in water/SDS media were obtained from 200ms NOESY experiment were identified. Spectrum crosspeaks were assigned, integrated and classified accordingly as strong, medium, weak and very weak (intensity thresholds). Distance calibration was done using the bin method by assigning an upper limit distance to in each peak intensity class: 2.7 Å for strong nOes, 3.4 Å medium, 4.5 Å weak and 6Å to very weak.In the end, 79 (33 sequential and 46 non-sequential) distance restraints (see list below) were introduced into our simulated annealing protocol.

```
! nOe restraints from both kf_Ca_2.xpk & kf_NH_2.xpk calibrated by bin method
! restraint for cycle (residue 9-14)
! manually generated ( last update 21.04.05)
! methyls scaled by .3
! Upper bounds for restrains have been modified.
! Strong >80 , Medium >40, Weak >20, very weak
!       <2.5A ,      <3.6A,    <4.8A,       <6A
! restrains=upper limits
! Performance note= seems to converge pretty well.  (60% success / before trying to correct minor violations)
assign (resid 11 and name HA) (resid 13 and name HN) 6.000  6.000 0.50 !     kf_noe200_cycle_NH.68 mod lowr bin
assign (resid 11 and name HB) (resid 13 and name HN)  6.00 6.000  0.50 ! kf_noe200_cycle_NH.69 modified to lwr bin
assign (resid 11 and name HG2# or resid 11 and name HG1#) (resid 13 and name HN) 6.000 6.0 0.50 ! kf_noe200_cycle_NH.71
assign (resid 14 and name HN) (resid 13 and name HN) 2.500  2.500 0.50 !    kf_noe200_cycle_NH.109
assign (resid 12 and name HN) (resid 13 and name HN) 3.600 3.600  0.50 !    kf_noe200_cycle_NH.78
assign (resid 12 and name HA) (resid 13 and name HN) 2.0 2.0 0.50 !    kf_noe200_cycle_NH.88
assign (resid 14 and name HA) (resid 13 and name HN) 4.80 4.80 0.50 !    kf_noe200_cycle_NH.108
assign (resid 11 and name HN) (resid 10 and name HN) 2.00 2.00  0.50 !    kf_noe200_cycle_NH.77
assign (resid 9 and name HA) (resid 10 and name HN) 3.600 3.600 0.50 !     kf_noe200_cycle_NH.28
assign (resid 9 and name HB) (resid 10 and name HN) 3.600 3.600 0.50 !     kf_noe200_cycle_NH.27
assign (resid 9 and name HG2#) (resid 10 and name HN) 4.800 4.800 0.50 !   kf_noe200_cycle_NH.30
assign (resid 11 and name HA) (resid 10 and name HN) 4.800 4.800 0.5 !    kf_noe200_cycle_NH.72
assign (resid 9 and name HB) (resid 11 and name HN) 4.800  4.800 0.50 !      kf_noe200_cycle_NH.20
assign (resid 9 and name HG2#) (resid 11 and name HN) 6.00  6.00 0.50 !      kf_noe200_cycle_NH.21
assign (resid 10 and name HA) (resid 11 and name HN) 2.500 2.500 0.50 !    kf_noe200_cycle_NH.36
assign (resid 14 and name HN) (resid 12 and name HN) 4.80  4.800 0.50 !    kf_noe200_cycle_NH.105 !
assign (resid 11 and name HA) (resid 12 and name HN) 2.00 2.0 0.50 !    kf_noe200_cycle_NH. manually generated
assign (resid 11 and name HB) (resid 12 and name HN) 2.500 2.50 0.50 !    kf_noe200_cycle_NH.55
assign (resid 14 and name HG2# or resid 14 and name HG1#) (resid 12 and name HN) 4.80 4.80 0.50 ! kf_noe200_cycle_NH.101
assign (resid 12 and name HA) (resid 14 and name HN) 3.600 3.600 0.0 !     kf_noe200_cycle_NH.83 !
assign (resid 10 and name HA) (resid 14 and name HN) 4.800 4.800 0.50 !     kf_noe200_cycle_NH.42
assign (resid 11 and name HA) (resid 14 and name HN) 3.600 3.600 0.50 !     kf_noe200_cycle_NH.61 !
assign (resid 14 and name HN) (resid 11 and name HN) 4.800 4.800 0.50 !    kf_noe200_cycle_NH.106
assign (resid 12 and name HB1 or resid 12 and name HB2) (resid 14 and name HN) 6.00 6.00 0.50 !    kf_noe200_cycle_NH.84
assign (resid 9 and name HG2#) (resid 14 and name HN) 6.000 6.000 0.50 !         kf_noe200_cycle_NH.24
assign (resid 11 and name HB) (resid 14 and name HN) 6.000 6.000 0.50 !     kf_noe200_cycle_NH.62
assign (resid 9 and name HB) (resid 14 and name HN) 6.000 6.000 0.50 !         kf_noe200_cycle_NH.23
```

```
! nOe restraints from both kf_Ca_2.xpk & kf_NH_2.xpk calibrated by bin method
! restraint for cycle (residue 9-14)
! Ricard 07.04.05 manually generated
! methyls scaled by .3
! Strong >80 , Medium >40, Weak >20, very weak
!       <2.5A ,      <3.6A,    <4.8A,       <6A
assign (resid 14 and name HG1# or resid 14 and name HG2#) (resid 10 and name HA) 4.8 4.8  0.50 !   kf_noe200_Ca.37/38
assign (resid 14 and name HA) (resid 11 and name HA) 6.00 6.00  0.50 !     kf_noe200_Ca.2  manual intensity
assign (resid 14 and name HG1# or resid 14 and name HG2#) (resid 11 and name HA) 4.800 4.8 0.50 !   kf_noe200_Ca.31/32
assign (resid 9 and name HB) (resid 14 and name HA) 4.800 4.8 0.50 !      kf_noe200_Ca.22
assign (resid 14 and name HG2# or resid 14 and name HG1# ) (resid 12 and name HB#) 6.00 6.0 0.50 !          kf_noe200_Ca.84/83
assign (resid 14 and name HB) (resid 12 and name HB1 or resid 12 and name HB2) 6.000 6.0 0.50 !          kf_noe200_Ca.80/89
assign (resid 9 and name HG2#) (resid 14 and name HA) 6.00 6.0 0.50 !      kf_noe200_Ca.manual peak Inoe=45

! nOe restraints from  noesy Tm=200ms calibrated by bin method
! restraint for khf tail (residue 1-8))
! manually generated ( last update 13.04.05)
! methyls scaled by .3
! Upper bounds for restrains have been modified.
! Strong >80 , Medium >40, Weak >20, very weak
!       <2.5A ,      <3.6A,    <4.8A,       <6A
! restrains=upper limits
assign (resid 1 and name HA1 or resid 1 and name HA2) (resid 2 and name HN) 2.500 2.500 0.50 !      kf_noe200_NH_s_tail.8
assign (resid 3 and name HA or resid 3 and name HB) (resid 2 and name HN) 4.800 4.8 0.50 !       kf_noe200_NH_s_tail.12 assingment
assign (resid 4 and name HA) (resid 2 and name HN) 6.000 6.00 0.50 !       kf_noe200_NH_s_tail.14
assign (resid 4 and name HA) (resid 3 and name HN) 4.800 4.800 0.50 !      kf_noe200_NH_s_tail.17
assign (resid 4 and name HN) (resid 3 and name HN) 2.500 2.500 0.50 !      kf_noe200_NH_s_tail.19
assign (resid 2 and name HN) (resid 3 and name HN) 3.600 3.6 0.50 !       kf_noe200_NH_s_tail.18
assign (resid 2 and name HA) (resid 3 and name HN) 2.500 2.500 0.50 !      kf_noe200_NH_s_tail.20 v.s. overlap/overestimated
assign (resid 2 and name HN) (resid 4 and name HN) 6.000 6.000  0.50 !      kf_noe200_NH_s_tail.27
assign (resid 5 and name HA) (resid 4 and name HN) 6.000 3.0 0.50 !       kf_noe200_NH_s_tail.32 weak/ no upper limit
assign (resid 1 and name HB1) (resid 4 and name HN) 6.00 6.0 0.50 !      kf_noe200_NH_s_tail.29
assign (resid 1 and name HG) (resid 4 and name HN) 6.00 6.0 0.50 !      kf_noe200_NH_s_tail.31 assignment s.c.
assign (resid 7 and name HN) (resid 5 and name HN) 6.000 6.000 0.50 !      kf_noe200_NH_s_tail.42
assign (resid 4 and name HN) (resid 5 and name HN) 4.800 4.800 0.50 !      kf_noe200_NH_s_tail.38 close to diagonala bin mod
assign (resid 4 and name HA) (resid 5 and name HN) 2.500 2.50 0.50 !       kf_noe200_NH_s_tail.39
assign (resid 6 and name HA) (resid 5 and name HN) 6.000 6.00 0.50 !       kf_noe200_NH_s_tail.43 bin limit
assign (resid 8 and name HB) (resid 5 and name HN) 4.800 4.800 0.50 !      kf_noe200_NH_s_tail.36
assign (resid 8 and name HN) (resid 7 and name HN) 2.500 2.500 0.50 !      kf_noe200_NH_s_tail.52
assign (resid 6 and name HA) (resid 7 and name HN) 2.500 2.50 0.0 !       kf_noe200_NH_s_tail.53 v.s.
assign (resid 5 and name HA) (resid 7 and name HN) 6.000 6.0 0.50 !       kf_noe200_NH_s_tail.57
assign (resid 6 and name HB2 or resid 6 and name HB1) (resid 7 and name HN) 3.600 3.6 0.0 !      kf_noe200_NH_s_tail.55 & 59
assign (resid 6 and name HD1 or resid 6 and name HD2) (resid 7 and name HN) 6.0 6.0 0.0 !      kf_noe200_NH_s_tail.56 m.b
assign (resid 6 and name HB1 or resid 6 and name HB2) (resid 8 and name HN) 4.8 4.8  0.50 !      kf_noe200_NH_s_tail.69
assign (resid 5 and  name HB) (resid 8 and name HN) 4.800 4.8 0.50 !      kf_noe200_NH_s_tail.70 overlapping/overestimation?
assign (resid 6 and name HD1 or resid 6 and name HD2) (resid 8 and name HN) 6.000 6.0 0.50 !      kf_noe200_NH_s_tail.63/64
assign (resid 6 and name HA) (resid 8 and name HN) 3.600 3.6 0.50 !       kf_noe200_NH_s_tail.67 overlapping/overestimation?
assign (resid 5 and name HG1# or resid 5 and name HG2#) (resid 8 and name HN) 4.800 4.8 0.50 !     kf_noe200_NH_s_tail.61

! nOe restraints from  noesy Tm=200ms calibrated by bin method
! restraint for khf tail Ha region (residue 1-8))
! manually generated ( last update 19.04.05)
! methyls scaled by .3
! Upper bounds for restrains have been modified.
! Strong >80 , Medium >40, Weak >20, very weak
!       <2.5A ,      <3.6A,    <4.8A,       <6A
! restrains=upper limits
assign (resid 1 and name HB#) (resid 2 and name HA) 6.0 6.0 0.50 !      kf_noe200_Ca.62
assign (resid 2 and name HA) (resid 3 and name HA) 4.800 4.8 0.50 !       kf_noe200_Ca.6 assgmnt HA/HB
assign (resid 4 and name HA) (resid 3 and name HA) 6.00 6.0 0.50 !       kf_noe200_Ca.7 assgmnt HA/HB
assign (resid 6 and name HD1 or resid 6 and name HD2) (resid 4 and name HA) 6.00 6.0 0.0 !      kf_noe200_Ca.12
assign (resid 6 and name HG1 or resid 6 and name HG2) (resid 4 and name HA) 6.00 6.0 0.50 !      kf_noe200_Ca.53
assign (resid 8 and name HB) (resid 4 and name HA) 6.0 6.0 0.40 !      kf_noe200_Ca.55 assgnt changed
assign (resid 1 and name HB#) (resid 4 and name HA) 6.0 6.0 0.50 !      kf_noe200_Ca.56
assign (resid 1 and name HG) (resid 4 and name HA) 6.0 6.0 0.50 !      kf_noe200_Ca.57
assign (resid 6 and name HD1 or resid 6 and name HD2) (resid 5 and name HA) 2.500 2.5 0.50 !      kf_noe200_Ca.9
assign (resid 4 and name HA) (resid 5 and name HA) 4.8 4.8 0.0 !       kf_noe200_Ca.11 close to diagonal mod. bin
assign (resid 8 and name HB) (resid 5 and name HA) 6.0 6.0 0.50 !      kf_noe200_Ca.45 assgnt changed
assign (resid 5 and name HG1# or resid 5 and name HG2#) (resid 6 and name HD#) 3.60 3.6 0.50 !    kf_noe200_Ca.75
assign (resid 8 and name HB) (resid 6 and name HD1 or resid 6 and name HD2) 6.0 6.0 0.50 !      kf_noe200_Ca. manual
```

```
assign ( resid 8 and name HA) (resid 5 and name HA) 6.0 6.0 0.50 !       kf_noe200_Ca.16 overlapping w 7HA.

! nOe restraints from  noesy Tm=200ms calibrated by bin method
! restraint for khf Ha specially residues linkingn tail and cycle)
! manually generated ( last update 18.04.05)
! methyls scaled by .3
! Upper bounds for restrains have been modified.
! Strong >80 , Medium >40, Weak >20, very weak
!       <2.5A ,      <3.6A,    <4.8A,       <6A
! restrains=upper limits
assign (resid 14 and name HG2# or resid 14 and name HG1# ) (resid 8 and name HA) 4.800 4.8 0.50

! nOe restraints from  noesy Tm=200ms calibrated by bin method
! restraint for khf specially residues linkingn tail and cycle)
! manually generated ( last update 25.04.05)
! methyls scaled by .3
! Upper bounds for restrains have been modified.
! Strong >80 , Medium >40, Weak >20, very weak
!       <2.5A ,      <3.6A,    <4.8A,       <6A
! restrains=upper limits
assign (resid 7 and name HB1) (resid 9 and name HN) 6.0 6.0 0.50 !      kf_noe200_NH_s_tail.86
assign (resid 5 and name HB) (resid 9 and name HN) 6.000 6.0 0.50 !      kf_noe200_NH_s_tail.83 reassigned
assign (resid 8 and name HA) (resid 9 and name HN) 2.500 2.5 0.50 !       kf_noe200_NH_s_tail.79 v.s ( overestimated?)
assign (resid 8 and name HG1#) (resid 10 and name HN) 6.000 6.0 0.50 !      kf_noe200_NH_s_tail.2
assign (resid 8 and name HG1#) (resid 11 and name HN) 6.000 6.0 0.50 !      kf_noe200_NH_s_tail.5
```