UNIVERSITY OF KWAZULU-NATAL

PROLINE *N*-OXIDE: MANIPULATION OF THE 3D CONFORMATION OF LINEAR PEPTIDES

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A thesis submitted to the College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Science.

As the candidate's supervisors, we have approved this thesis for submission.

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LIST OF ABBREVIATIONS

CTC: 2-Chlorotrityl chloride

DCM: dichloromethane

DIEA: *N*,*N*-Diisopropylethylamine

DMF: dimethyl formamide

DMP: dimethylphosphinic acid

DMSO: dimethyl sulfoxide

Fmoc: fluorenylmethyloxycarbonylchloride

HBTU: *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate

m-CPBA: meta-Chloroperoxybenzoic acid

MSA: methanesulfonic acid

TFA: trifluoroacetic acid

TFE: trifluoroethanol

THF: Tetrahydrofuran

ABSTRACT

During the last the few years, the number of peptide pharmaceutical drugs reaching the market has notably increased. Drug based peptides have many advantages, such as high potency of action and limited off-target side effects that are not present in most small molecules. However, oral bioavailability of peptides is a major obstacle that hinders the development of more therapeutic formulations. Physiochemical properties of peptides, such as short plasma half-life, sensitivity to enzyme degradation and the tendency to undergo aggregation, are some of the main reasons for the lack of bioavailability. Enhancement of bioavailability can be achieved when the flexibility of peptides is reduced to a more defined stereochemical structure. There are some approaches that can reduce the flexibility of peptide backbones to a more defined structure *e.g.* application of peptidomimetics and cyclic peptides. Here, we have investigated another new tool that can lead us to the same goal.

The purpose of this study was to investigate the possibility of modulating the secondary structure of short peptides. This was envisaged *via* the application of a proline *N*-oxide moiety and by *N*-methylation of the peptide backbone. The major objectives for this study were:

1. To synthesise a series of tetrapeptides to investigate the effect of proline *N*-oxide on their secondary structure.
2. The effect of *N*-methylation of the amide bond on isomerization of the *N*-oxide peptide backbone in terms of *cis*- and *trans*-isomers and how it directs the potential hydrogen bonding interactions
3. To study the effect of side chain bulkiness on the potential of hydrogen bonding interactions of the selected peptides.
4. Analyses of the formed secondary structures with advanced NMR techniques.
5. To perform a thermal coefficient NMR study on these molecules in order to obtain an improved understanding of the nature of hydrogen bond interactions of these peptides.

DECLARATION

I, **Majid Darestani Farahani** declare that:

1. The research reported in this thesis, except where otherwise indicated is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from that person.
4. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the reference sections.

Signed

_____

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I sing endless praises to God for allowing me to see the end of this, He is forever faithful, loving and gracious, He is a friend that sticks closer than a brother and His name is worthy to be praised.

I would like to thank my wife (Saba Alapour) for her unique, kind and constant support. Thanks also to my father (Mohammadreza Darestani Farahani) and mother (Sadigheh Darestani Farahani) for their advice, encouragement and the manner in which they helped me grow into the person I am today.

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Chapter 1

1 Introduction

1.1 Peptides

Peptides are short polymers formed from linking amino acids in a defined order.[1] Ordinary amide bonds play the role of connector between these building blocks to form a long backbone.[2] To join these building blocks to each other through amide bonds, an essential step is activation of the amine in one amino acids and the carboxylic group in the second one, followed by amide/peptide formation. A peptide normally involves up to 50 amino acids, while biopolymers with more than 50 amino acids are called polypeptides/proteins.[3] Natural amino acids largely exist in alpha (α) forms, these being organic molecules, with a carboxylic acid on one side of the central α-carbon and an amine group on the other.[4] In most cases one R-side chain is also attached to this carbon. For the simplest natural amino acid, glycine, R = H. It therefore is the only achiral amino acid in nature (**Figure 1.1**).[5]

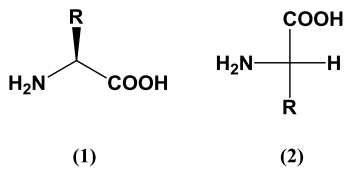


Figure 1.1. Illustrative representations of natural amino acids: (1) α-amino acid and (2) the Fischer representation

These chiral amino acids have non-superimposable mirror images, also called enantiomers. Enantiomers have identical chemical and physical properties in the absence of an external chiral influence. This means that both will have the same melting points, solubility, chromatographic retention time, IR and NMR spectra. If the two enantiomers are mixed, the resulting sample will have different physical properties, such as melting point and solubility, but the chemical based properties such as chromatographic, IR and NMR behaviour will be unchanged. This has an important consequence if we want to determine the proportion of the two enantiomers in a mixture: standard chromatographic and NMR methods must be modified to introduce an external chiral influence. Only then will the enantiomers behave differently from each other and analysis be possible.[6,7]

There is one property in which enantiomers differ and that is the direction in which they rotate the plane of plane-polarized light. This phenomenon of optical activity provides the basis for the nomenclature of enantiomers. The enantiomer which rotates plane-polarized light in a clockwise direction ($[\square]_D$ = +14.6° in 1.5 M HCl) is denoted (+)-alanine while the other which has an equal and opposite rotation ($[\mathbf{a}]_D$ = -14.6°) is denoted (-)-alanine. Since the rotations due to the individual enantiomers are additive, the net measured rotation may be used as a guide to the enantiomeric composition (provided the individual rotations are known).

This (+) - (-) nomenclature must not be confused with the (*R*) and (*S*) system (Cahn-Ingold-Prelog).[8] Only the latter nomenclature enables us to describe the three-dimensional structure of a chiral compound. Although the Fisher (*L* and *D*) system is still used in Biochemistry it should not be confused with the (+) for Levo and (-) for Dextro. There is no correlation between the (+), (-) and *L*, *D*. All natural occurring α-amino acids are (S)-forms (or L-forms) with the exception of cysteine, which is normally in (*R*)-from. Amino acids are categorized into five different classes: acidic, neutral, basic, hydrophobic and hydrophilic.[9]

1.2 Three dimensional (3D) structures of peptides

Peptides or proteins have primary, secondary, tertiary and quaternary structures that contribute to the overall 3D structure. Proteins and peptides illustrate remarkable and highly variant biological activities due to the ability of these intrinsically bendable chains to fold into well-ordered and compact secondary and tertiary structures.[1] Linus Pauling, approximately half a century ago, realized that the unravelling of the secret interactions of these super molecules in biological processes and living cells depends on optimal information of their 3D structures.[10] A small distinct change in secondary formations such as helices, sheets and turns, yield different configurations of tertiary and quaternary structures.[11] A nomenclature has been defined for every peptide conformation with respect to their type of hydrogen bonds.[12] However, there are several unique structures that do not fit in this categorization and called statistical coil.[13-15]

1.2.1 Primary structure of peptides

Generally, polypeptides or proteins are linear or unbranched polymers, which enables their primary configurations to be frequently identified in terms of the amino acids along their backbones. Proteins are

often cross-linked (most often by disulfide bonds). In addition, the primary structures also demand specifying the cross-linking atoms particularly when cysteine is involved in disulfide bonds.[16]

1.2.2 Secondary structure of peptides

Due to the existence of different amino acids in a peptide/protein with various side chains and functional groups, the linear form of the peptide/protein is generally not the most stable conformation. Normally, stabilization occurs through hydrogen bonding interactions forming secondary structures such as helices, sheets and turns.

1.2.2.1 Helices

Helices are formed with regular folding and present as a corkscrew shape such as α-helix and 3.10 helix. In the majority of examples, peptides with this secondary structure prefer to turn clock-wise and form a right-handed helix. This family can be differentiated into a few subcategories, depending on the periodicity of the helix.[17] The α-helix is the most common configuration and its characteristics include first, the existence of a hydrogen bond between carbonyl group of residue (i) and amide of residue (i + 4) that presents a 13 membered ring. Second, each loop has a length of 0.54 nm and involves 3.6 amino acid segments. Third, the measured dihedral angels, Ψ and Φ are between 45 to 60 degrees. This type of secondary structure is typically very densely packed and all the side chain of the amino acids point out of the helix (**Figure 1.2**).[2]

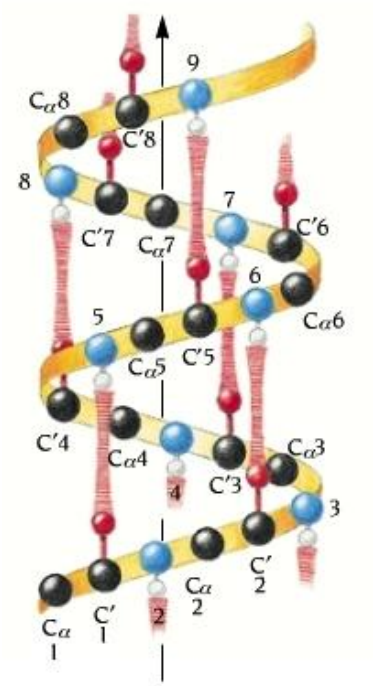


Figure 1.2. α-helix[18]

1.2.2.2 β-Sheet

A β-Sheet is a commonly observed secondary structure in proteins or biopolymers, this being a plainer conformation that two residues being adopt it. Similar to other types of secondary formations of peptides, hydrogen bonds play a critical role. There are two different classes of β-sheets and both are formed through intermolecular hydrogen bonding interactions between at least two backbones (**Figure 1.3**).[2] These are the parallel β-sheet and parallel β-sheet. The residues for the first are oriented in the same direction for both backbones, but the direction of the fragments for the latter are opposite.

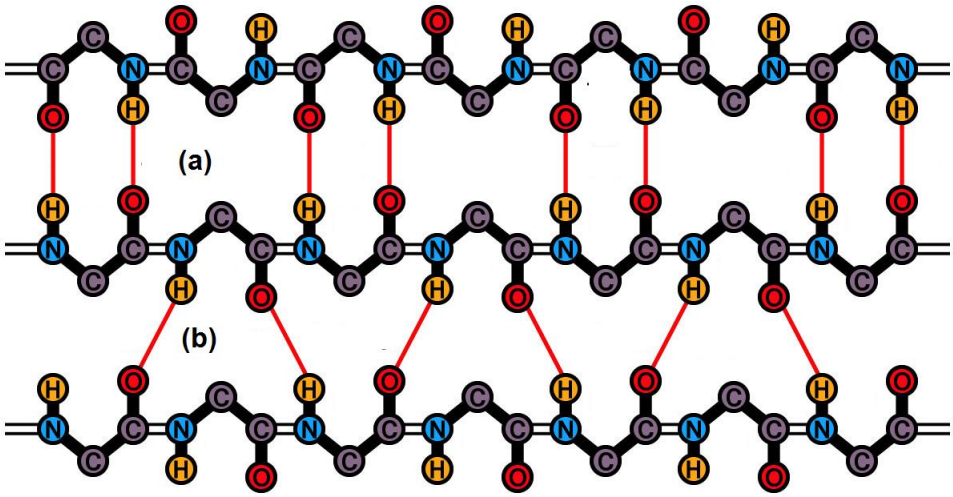


Figure 1.3. (a) Anti-parallel β-sheet, (b) parallel β-sheet[19]

1.2.2.3 Turns in peptides and proteins

Turns are known as small secondary structures that form an "elbow" in peptides and in some cases can induce intramolecular β-sheet formation with two dominating fragments. The internal hydrogen bonds stabilize the peptide backbones and the size of the "rings" is the basis for classification. Different types of turns are categorized according to number of amino acids involved in the turn systems leading to δ-turns (two amino acids) γ-turns (three amino acids), β-turns (four amino acids), α-turns (five amino acids) and π-turns (six amino acids).[20]

The β-turn is the most important type due to its ability to influence the biological activity of a peptide. This advantage appears to originate from an improved fitting of peptide with this type turn as a ligand to the pocket of an enzyme or any other type of receptor. β-turns are characterised by the hydrogen bonding interaction between the residue (i) and residue (i + 3) in the same backbone. Depending on the different dihedral angles that this bond can form in the sequence, ten types (I, I′, II, II′, III, III′, IV, V, VI and VII) of β-turns may exist (**Figure 1.4**).[2,21]

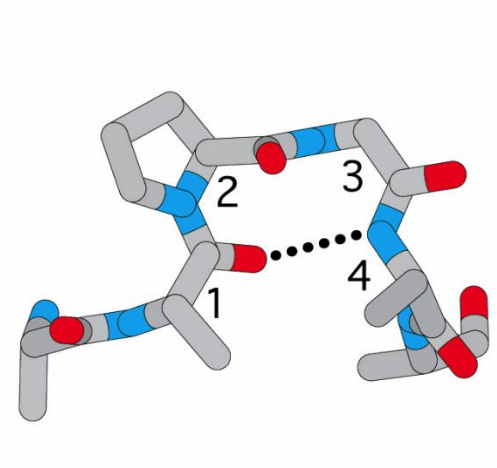


Figure 1.4. A schematic representation of β-turns[22]

All amino acids, with the exception of proline form peptides where the amides are mainly observed as *trans*-isomers.[23-29] This bond arrangement has a profound effect on the secondary structure. The presence of proline in peptide sequence offers the possibility of *cis/trans*-isomerization of amide bond at the *N*-terminal part of proline.[20] This process is known as the fundamental origin for peptide refolding in X-Pro peptides.[30-39] Secondary structures can be organized by controlling the isomerization in these types of super molecules and the inclusion of amino acids with aromatic residues in the peptide backbone is a common technique to tune the *cis/trans*-isomerization through π-π and other electrostatic interactions.[23,24,40-45]

More discussion on the secondary structure of peptides/proteins following in Chapter 2.

1.2.3 Tertiary structure of peptides

The tertiary structure is the final specific geometric shape that a protein assumes and is defined as a three-dimensional conformation of a particular protein fragment. The β-sheets and α-helices prefer to form compact globular structures. Folding is controlled with the non-specific hydrophobic interactions, but the structure is fixed when parts of the protein domain are locked into place by specific tertiary interactions such as, hydrogen bonds, salt bridges, tight packing of side chains and disulfide bonds. Disulfide bonds are extremely infrequent in cytosolic proteins, as a reducing surrounding is normally present in the cytosol.[46]

1.2.4 Quaternary structure of peptides

Quaternary structures of peptides are the result of the arrangements of at least two folded or coiling protein molecules to form a single multi-protein complex where the individual units are kept together by weak intermolecular interactions. Enzymes frequently form quaternary structures, for example the HIV protease consists of a homodimer where each of the C2 symmetric subunits consists of 99 amino acids. Holoenzymes form a class of enzymes with very much regular subunits of which the functional core is known as the catalytic subunit.[47]

During the last decade, the number of peptide-based drugs approved by the Food and Drug Administration (FDA), USA, increased considerably.[48-52] These molecules have attracted scientist's attention due to advantages, such as *e.g.* high potencies of action and limited off-target side effects.[53]

However, beside their benefits, some disadvantages prevent their more wide application in pharmaceutical science. Low oral bioavailability and pharmacokinetic instability are the chief factors that have hindered the progress and development of peptides in the medicinal chemistry field of science.[54,55] The reason for these drawbacks originates from their physiochemical profile, such as their sensitivity to enzyme degradation, short plasma half-life.[56-58] Peptide chemists have been attempting to eliminate these weak points with diverse strategies that will be reviewed in this chapter.

1.3 Peptidomimetics

The goal of peptidomimetics is to control the spatial disposition of amino acid functional groups that simulate a peptide structure through alternative molecules.[59-62] Peptidomimetics inherit characteristics of their parent structures, but offer improved efficacy and stability in *vitro* against bacterial, fungal and eukaryotic origins.[63] Application of these designed oligomers replicates or mimics is seen as an valuable tool for future drug discovery.[64]

1.3.1 *N*-Methylated peptides

One example of a widely used peptidomimetic approaches is the use of *N*-methylated amino acids. This phenomenon also occurs in nature and there are well known examples of multiple *N*-methylated natural cyclic peptides,[65] such as omphalotin, cyclosporine, (**Figure 1.5**) with important pharmacological and biological properties.

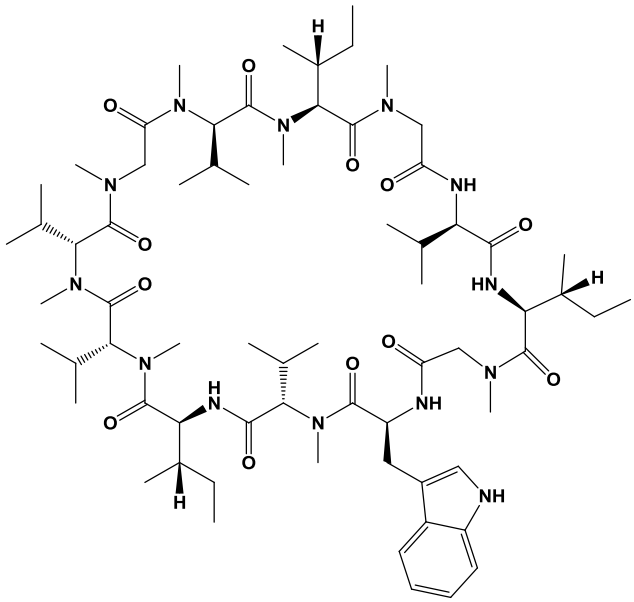


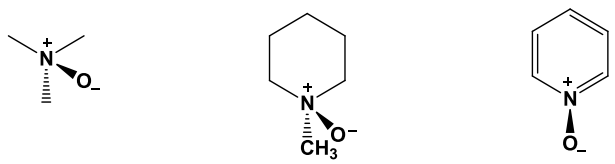
Figure 1.5. Structure of omphalotin-A

Chatterjee *et al.*[66] reported that multi *N*-methylation in peptides not only increases receptor subtype selectivity, but also improves the oral bioavailability of the peptide.[67] It has been reported that the *N*-methylation of at least every second amino acid prevents the occurrence of proteolytic degradation on the peptide backbones.[68,69] Poly *N*-methylated peptides have shown promising results in reducing the general cytotoxicity of peptide drugs due to the toxicity of degraded products.[68] In a number of studies, the pharmacological properties of peptides have been altered through the application of *N*-methylation for a single amino acid.[70]

1.4 Chemistry of *N*-oxides

N-oxides are organic compounds with N-O$^-$ bonds and this bond is known to exhibit unusual dipolar covalent nature.[22] The nitrogen atom can be sp^2 (heteroaromatic nitrogen) or sp^3 hybridized (aliphatic nitrogen) depending on the structure.[19] *N*-oxide derivatives were first discovered as the oxidation products of tertiary amines before 1900.[18]

There are three types of *N*-oxides, which differ in substituents that are bonded to the nitrogen atom (**Figure 1.6**).[18] Their biological importance and applications as intermediates in organic synthesis motivated scientists to investigate these molecules.[71] Heterocyclic *N*-oxides have also been utilized as ligand catalyst, auxiliary agents, protecting groups and oxidants.[18,72]



(1) (2) (3)

Figure 1.6. Three general forms of *N*-oxide compounds: (1) Heteroaliphatic *N*-oxide, (2) heterocyclic *N*-oxide, (3) heteroaromatic *N*-oxide

1.5 Analysis and conformational studies of peptides

There are many approaches to investigate the secondary structures of peptides, the most common and efficient techniques being Nuclear Magnetic Resonance (NMR), Infrared (IR), Circular Dichrosim (CD), X-Ray Diffraction (XRD) and computational methods. These are briefly outlined in the following sections.[73-76]

1.5.1 NMR studies

As discussed previously, hydrogen bonds are responsible for forming secondary structures in the peptide backbone[77] and the chemical shifts of amide protons to the downfield region of the ^{1}H NMR spectrum are useful for the determination of peptide organization.[78] NMR active nucleuses are defined for isotope of elements that do not present nuclei with spin quantum number of zero. $^{1}H_{1\ and}\ ^{13}C_{6}$ are two commonly nuclei with nuclei spin quantum numbers of ½ and are used in NMR spectroscopy. $^{15}N_{7}$ has the same nuclei spin as $^{1}H_{1\ and}\ ^{13}C_{6}$ but its signal is weak due to low natural abundance of this isotope for nitrogen. In addition, ^{15}N NMR experiments are very helpful tools as nitrogen atoms are present in each residue of these biopolymers but the isotope enriched conditions should be used for the synthesis of biosynthesis of such systems.[79] Another valuable tool is the Nuclear Overhauser Effect (NOE). This experiments give through space proton-proton interactions and thus allow the observer to determine the distance between two nuclei up to about 5-6 A°.[80]

1.5.2 Choice of solvent

Solvent selection is important in NMR studies for these molecules. Since peptides are amphiphilic in nature, the polarity of the solvents has a profound influence on the conformation of peptides. When a polar solvent is utilized, the non-polar groups in the peptide are concealed inside the backbone and the peptides with more polar groups are exposed to the surface/solvent. The opposite occurs when apolar solvents are employed. The solubility of peptides depends on their sequences. Typical apolar solvents

are Benzene-d_6, $CDCl_3$ and polar solvents such as DMF-d_7, DMSO-d_6, MeOD and D_2O are often used. Due to the high dependence of peptide secondary structure to its environment, numerous conformations can be achieved by varying the solvent.[73]

1.5.3 2D NMR techniques[20]

2D NMR spectra offer valuable data that make them a powerful tool for analysing and detecting secondary structures in peptides. In order to detect an unlabelled protein using this technique, a full set of two-dimensional NMR experiments is necessary. When larger systems are analysed, it is much more convenient to use ^{15}N labelled sources in the (bio) synthesis of the protein, as 2D correlations between the nitrogen and protons in close proximity (^{15}N HSQC)[81] provides invaluable information about the structure of the peptide/protein. For a better understanding of application of each NMR experiment, a brief report is provided. Note that the first methods all make use of through bond correlations, while the NOE related methods make use of through space correlations that is helpful for estimation of 3D structures.

1.5.3.1 **COSY:** (Homonuclear Correlated Spectroscopy).

The cross peaks in this experiment appear whenever the spin resonance at δ_1 and δ_2 are coupled to each other.[82,83] Many advanced COSY based NMR experiments have been invented, such as DQFCOSY (double-quantum filtered), [84] COSY45,[85] LR COSY[86] and ECOSY.[87]

1.5.3.2 **TOCSY:** (Total Correlation Spectroscopy).[82]

This experiment utilizes a spin-lock for coherence transfer. This process changes the coupled system to 'strongly coupled', leading to cross peaks between all resonances in a coupled system. Signal detection of weak coupling that is not detected in ordinary COSY can then be observed.

1.5.3.3 **INADEQUATE:** (Incredible Natural Abundance Double Quantum Transfer Experiment).[88]

2-Dimensional J_{C-C} is a double quantum coherence experiment that produces a C-C correlation using $^1J_{CC}$. This experiment is probably the most powerful tool for structure-determination when compare to all others, but suffers from very low sensitivity.[89] This disadvantage originates from low one-bond carbon-carbon coupling constant that ranges from 30 to 80 Hz, approximately.[80]

1.5.3.4 CH-COSY or HETCOR: (Heteronuclear Correlation).[108]

^{1}H and ^{13}C correlations give rise to unique coupling constants, denoted by J_{C-H}. In this method, vicinal ($^{1}J_{C-H}$) or geminal ($^{2}J_{C-H}$) or long rang couplings $^{3}J_{C-H}$ are detected. Sensitivity is poor due to low population of the ^{13}C nuclease, and it has been widely replaced with advanced methods such as HMQC and HMBC.[90,91]

1.5.3.5 ^{13}C HSQC: (^{13}C isotope Heteronuclear Single Quantum Coherence).[90]

This method is a CH correlation experiment and is used to detect proton signals attached to ^{13}C atoms. It displays higher resolution in the C-dimension than does the related HMQC experiment, if the pulse-calibration was done optimally. See the comment in section 1.5.3.7 as well.

1.5.3.6 ^{15}N HSQC: (^{15}N isotope Heteronuclear Single Quantum Coherence).[92]

This method is similar to ordinary HSQC except for using the natural abundance of the ^{15}N isotope instead of ^{13}C. This experiment is one of the most important techniques for the investigation of secondary structures of peptides and proteins due to existence of NH for each residue with exception of proline.[92]

1.5.3.7 HMQC: (Heteronuclear Multiple Quantum Coherence).[90]

This method is very useful due to illustration of correlation between carbon and the attached hydrogen atom, similar to HSQC. The difference between the two techniques is that the evaluation time of an HMQC, both proton and X magnetization (e, g X=^{13}C) are allowed to evolve, whereas in HSQC only X magnetization is allowed to evolve.[90] Therefore, HSQC is very depended on the calibration of the NMR pulses, while HMQC is not.[90] When it is possible to correctly calibrate the NMR pulses, then HSQC will provide superior data.

1.5.3.8 HMBC: (Heteronuclear Multi-Bond Connectivity).[90]

This process acts similar to HMQC with the exception of not showing the one-bond correlations. It is normally used to detect magnetization transformation between proton and carbon of a molecule over two or three bonds.[93] This 2D experiment has shortcomings because the magnetization requires being transfer twice, which can create a delay in decreasing the peak signal intensity by usage of T_1 and T_2 relaxation. Note that C-X-H (X = N, S, O etc.) correlations are also observed.

1.5.3.9 NOESY, ROESY: (Proton-proton correlation mediated by dipolar coupling (NOE effect)).[82]

Both of these experiments are very powerful NMR protocols to determine 3D structures of compounds, which could be small molecules or proteins. It detects through space (intra and intermolecular) proton-proton interactions.

1.5.3.10 EASY-ROESY: (Efficient Adiabatic symmetrized Rotating Overhauser effect spectroscopy)[94]

Sometimes, in the case of medium size molecules (Mw around 1000 gr.mole^{-1}) the NOE correlation cannot be observed or the signal is very weak due to sign change of the NOESY. In such this case EASY-ROESY is a more reliable technique. This method is not only useful for measuring distances between two proton systems but can also be applied for measuring the exchange rate of a chemical reaction, if normal NOESY experiments cannot provide sufficient resolution.[94]

1.5.3.11 EXSY: (Exchange Spectroscopy- the 2D equivalent of the Forsen saturation transfer experiment).[85]

The transmission of magnetization is due to chemical exchange between A and B, rather than an NOE. EXSY uses an indistinguishable pulse sequence to the NOESY experiment, and is therefore subject to interference by NOE. The magnetization transfer is the equivalent of a negative NOE and it has a long mixing delay in comparison to NOESY.[85,95]

1.5.3.12 HOESY: (Heteronuclear Overhauser Effect Spectroscopy).[96]

Through space correlations between heteronuclei and protons that are close to each other can be detected with this experiment. The essential requirement for this NMR experiment is the significant relaxation for heteronuclei that can be provided with dipole-dipole interactions with nearby protons or other magnetic nucleus, enabling this heteronuclei to relax exclusively with failing of chemical shift anisotropy or quadrupolar mechanism. This method is extensively used in the spectroscopy of organolithium reagents.

The spin system in conventional correlation spectroscopy, COSY, is designed to transfer the α-proton magnetization between protons on the adjacent atoms. It is clear that an α-proton transfers magnetization to a β-proton and the β-proton transfers to an α- and γ-proton, if any are available, with the same occurring for γ-proton with β, δ-protons. This spin system is an invaluable tool to detect hydrogen atoms in α position, and simultaneously, the protons in the side chains of each amino acid residue in a peptide backbone. In order to connect the different spin systems, the application of NOESY or ROESY that

transfer magnetization through space seems to be necessary.[97] These experiments are able to show all cross peaks, which are close to each other with regards to the space, whether they are in the same spin system or not, provided that the molecule is rigid enough so that these interactions can be observed in the time scale of the NMR experiment. Therefore, NOESY and ROESY NMR experiments are powerful instruments for establishing close contacts between individual amino acids in a single peptide or protein backbone. These experiments have also been widely used to determine the secondary[13] structures of biopolymers. In most cases NOE data are provided to molecular dynamic experiments, in which these constraints are imposed to find the 3D structure of unknown proteins.[81]

1.5.4 Hydrogen deuterium exchange and NMR thermal analysis experiments

The involvement of protons in hydrogen bond interactions can be detected with both of these experiments. Utilization of solvents that offer exchangeable deuterium atoms to the dissolved peptide in a NMR tube, such as CD_3OD and D_2O/H_2O, are applied for this purpose. Access of amide protons to solvents is prevented or retarded by intramolecular hydrogen bonding interactions. The rate of disappearance of an amide proton peak is a measure of the strength of the hydrogen bond.[73,98,99]

The same principle is applicable for thermochemical investigations of these biopolymers. Generally, the amide proton involved in a strong hydrogen bonding interaction has a low temperature dependence coefficient ($-d\delta/dT < 3$ ppb/K), while for ordinary amide protons; this value is more than -5 ppb/K.[78,100-103]

1.5.5 Circular dichroism

Circular dichroism (CD) is based on optical activities of chiral molecules that rotate differential left- or right-handed circularly polarized light. Peptides are chiral molecules and rotate polarized light; the extent of rotation varies with wavelength. For peptides and proteins, the instrument is used to determine the basic conformation(s) present in macromolecules *e.g.* the secondary structure of peptides/ proteins or the handedness of DNA.[104] Although information from CD spectra is limited in comparison to data from NMR or X-ray diffraction, it can be conveniently utilized as a preliminary guide for the nature of secondary structures of peptides.[20,105] CD was invented by Aimé Cotton and the analysis is performed in highly dilute solutions (normally less than 1 mM, to avoid peptide aggregation) at the appropriate wavelength for UV absorption of the amide groups (180-230 nm).[106] This instrument measures the ellipticity of peptides with polarized UV light. Electron excitation of amide bonds ($\pi \rightarrow \pi^*$) can be observed and the absorption will vary based on the hydrogen or non-hydrogen bonded state of the amide.

This information provides reliable and valuable evidence about the shape of the secondary structure in the peptide backbone.[104] The technique readily provides information about the whether a peptide contains α-helix, β-sheet and statistical coil conformation, but it cannot tell which part of the peptide contains it.[105]

Peptides and proteins that possess few non-amino acid chromophores such as prosthetic groups do not display CD bands at wavelengths longer than 300 nm.[20] Normally, the most prominent chromophore of proteins and peptides responsible for the CD spectrum is the amide group.[20] There are two electronic transitions that can occur in the amide chromophore. The ($n \rightarrow \pi^*$) transition presents a weak signal and appears in a negative band around 220 nm. The energy (wavelength) of the amide ($n \rightarrow \pi^*$) transition is sensitive to hydrogen bond formation in the backbone.[20,105] The ($\pi \rightarrow \pi^*$)transition is much stronger and displays a signal as a positive band around 192 nm and a negative band around 210 nm.[20,105] Furthermore, the characteristics of an α-helical secondary structure include a negative band at 208 nm, and a positive band at 192 nm.[20,107] A β-sheet is characterized with a negative band at 216 nm and a positive band of comparable size close to 195 nm.[20,108] A statistical coil secondary structure is characterized with a strong negative CD band just below 200 nm.[20,108] These peaks can vary depending on the choice of solvent.

The sample chamber of this instrument is made from quartz with a path length ranging between from 0.1 to 10 mm.[109] Shorter path lengths are preferred to limit parasite noise generated from solvent absorption. The choice of solvent in this experiment is very important and the selected solvent needs to be UV inactive in the chosen band for the peptide. Solvents such as methanol (UV cut-off limit at 195 nm for a 1 mm cell), trifluoroethanol (TFE) and a mixture of methanol and water are common solvents for CD measurements. Other organic solvents *e.g.* THF, acetonitrile, chloroform or dichloromethane cannot be used for peptides of proteins due to overlap of UV absorption in the amide region.[104]

This technique is a fast method, as it can provide an immediate result about the secondary structure adopted by the peptides. A major disadvantage of this technique is that it cannot discriminate between amides that are, or are not involved in hydrogen bonding interactions. Nevertheless, experimental CD curves of α-peptides are typical of specific or mixtures of secondary structures[110] and observed peptide curves can be compared to published data. Many reports on CD spectra of β-peptides appear in literature but this approach has rarely been used to detect secondary structure of peptides that possess unnatural amino acids, as the CD spectra are likely to differ from peptides involving natural amino acids only.

1.5.6 IR spectroscopy in solution

Another interesting tool for the detection of intramolecular hydrogen bonds in peptides is to use IR in solution. In the IR spectrum, the non-hydrogen bonded amides usually present peaks in area of more than 3400 cm^{-1}, while hydrogen-bonded amides appear in area of less than 3400 cm^{-1}. The solvent selection is a determining factor for use of this method, as the application of solvents that are able to form hydrogen bonds with the substrate will induce interference.[111]

1.5.7 X-ray crystallography

This is the most powerful and exact method for detecting secondary structure in peptides. The only drawback factor for this technique is that crystal packing artifacts can cause deviation of the solution structure. Nevertheless, in the majority of cases the crystal structure compare quite well with computed or the real structure. It should just be noticed that large biomolecules are able to undergo dynamic movements and the crystal structure only present one specific ”snapshot”.[81] Unfortunately, obtaining the crystal structure of a peptide is difficult, especially when the peptide backbone is highly flexible.[13]

1.5.8 Computational studies

The 3D conformation of peptides can be obtained from NMR, CD and XRD studies. Another method that can supplement experimental results is molecular modelling of the peptide backbones. The molecular dynamics (MD) calculations have been reported as a pioneer method for this purpose.[112-115]

1.6 Thesis outline

The rest of the thesis discusses the synthesis of *N*-benzylproline *N*-oxide peptides and their analysis utilizing NMR methods. The aim of the study is to determine the structural behaviour of proline *N*-oxides incorporated into short peptides. The formed secondary structures of these biopolymers have been investigated. The strategy followed to unravel the effect of the *N*-oxide on the secondary structure was to make a logical comparison between the same peptide with, and without *N*-oxide modification. We have chosen two types of peptides for this purpose, one which is proposed to be more flexible and with amino acids that are known to induce turn structures, and a second that are less flexible and with amino acids that are known to prevent turn structures. In addition to that, selective *N*-methylation of amides bonds was also introduced to study the capability of *N*-oxide peptides to form quasi β-turn as is a potential valuable new type of secondary structure. Information about the conformations of the peptides was obtained from 2D NMR experiments (NOE) and thermal analysis of the ^{1}H NMR spectra of these molecules.

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CHAPTER 2

1 Proline *N*-oxide: Manipulation of the 3D Conformation of Linear Peptides

1.1 Introduction

During the last the few years, the number of peptide pharmaceutical drugs reaching the market has notably increased, as is indicated by the USA Food and Drug Administration's approval of six new peptide-based drugs in 2012.[1] This represents 15% of total new drug approvals, and peptides are starting to compete with small molecule drug medications (about 34% in 2012)[2] that dominated the market in the previous century. The recent success of peptide-based drugs is fuelling the interest of pharmaceutical companies and research groups in this field, which will lead to further development of peptides as therapeutics.[2-5] Drug based peptides have many advantages, such as high potency of action and limited off-target side-effects that are not present in most small molecules.[6] However, oral bioavailability of peptides is a major obstacle that hinders the development of more therapeutic formulations.[7,8] Physiochemical properties such as short plasma half-life, sensitivity to enzyme degradation and the tendency to undergo aggregation are some of the main reasons for the lack of bioavailability.[9-11] Enhancement of this property can be achieved when the flexibility of peptides is reduced to more defined stereochemical structures.[9-11]

The bioavailability of peptides can also be increased by selective *N*-methylation of some of the amide nitrogen atoms in the backbone,[12-14] as enzymes seem unable to hydrolyse such bonds.[15] In addition, *N*-methylation dramatically increases aqueous solubility of the peptide (as much as 1000 fold for one *N*-methylation).[15] However, a more remarkable result is that this modification simultaneously improves the lipophilicity,[13] proteolytic stability and conformational rigidity of these peptides.[12-14]

The amide bond of peptides containing natural amino acids appear mostly in the *trans*-conformation, with the exception of the amide bond at the *N*-terminal of proline.[16-21] In addition, proline also induces peptide folding, yielding secondary structures with β-turn or alpha helix character.[22-28] This

proline amide bond gives relatively high *cis-trans* isomerization (3-5% *cis*-isomers).[29] The availability of aromatic rings in normal peptides is known to be an important factor for an increase in the percentage of *cis*-proline isomers through intermolecular π-π interactions which severely restrict the conformational freedom of the peptides.[23,24,30-35] Studies have indicated that aromatic groups in short peptides can interact with proline through potentially tuneable aromatic π-electron donor/prolyl acceptor interactions.[16,36-41] The rotational energy barrier for proline *cis/trans*-isomerization is normally in range of 16-20 kcal/mol.[42] In some cases values as low as 8 kcal/mol have been recorded.[43,44] It appears that side chain π-π stacking contributes to this lower than usual rotational barrier. Changing the *cis/trans*-isomerization state can alter fundamental control of peptide folding.[30,45]

It is important to note that, energy barriers between 15-20 kcal/mol can be overcome at room temperature and for this reason proline is often used for folding of peptides.[46,47] Interestingly, individual cases have been reported where certain proline containing peptides gave up to 50% and also complete *cis*-conformations.[48] This isomerization is the rate-limiting step in the refolding of denatured proteins.[49-63] However, there is no definite rule with respect to the equilibrium of *cis*- and *trans*-isomers of peptides, but solvent polarity has an influence. A polar solvent, such as DMSO with a large dipolar moment (3.96 D), favours *cis*-isomers (K_{t-c} = 92 at 293 K), while less polar solvents, such as water (1.85 D), induce more *trans*-character (K_{t-c} = 151 at 293 K).[64,65] The isomerization ratio can be analysed with NMR spectroscopy from characteristic Nuclear-Overhauser (NOE) proton-proton distances.[49,66-68]

Replacement of the hydrogen on amides in peptides with a methyl group, will diminish hydrogen bonding interactions between peptide backbones,[15] which is responsible for β-sheet formation.[14,69-73] Other alterations of the secondary structure may also occur since *cis/trans* isomerization of the amide bond increases for *N*-methylation. This isomerization is sometimes used to regulate the function of the peptide.[74-77] As is the case for natural peptides there appears to be no particular rule that predicts or governs the ratio of *cis*- and *trans*-conformations of *N*-methylated peptides. These types of molecules generally present multiple *cis/trans* amide bond isomers, as is evident from recently reported NMR data.[17,68,78-84] Integration of the methyl groups in the proton spectra also reflected the *cis/trans* ratio.[78-80] In this type of ratio measurement, the experimental error

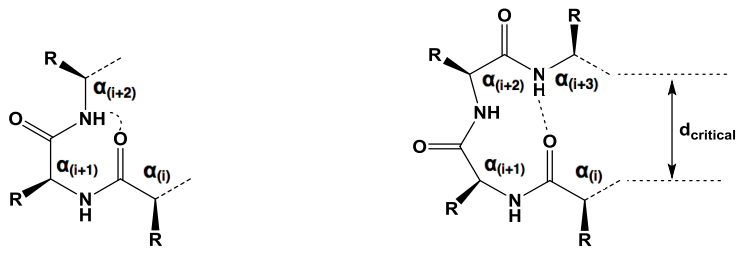
is approximately ±15 %.[78] According to previous reports on *N*-methylated peptides, the population of *cis*-isomers are generally higher at lower temperatures, but decrease at room temperature,[78,85] this equilibrium being highly depended on the local environment, such as peptide backbone or the folding thereof.[64] It was demonstrated in previous spectroscopic and computational studies on *N*-methylated polypeptides, that the ratio between the populations of *cis*- and *trans*-isomers varies and is highly depended on temperature, solvent and the type of amino acids in the backbone.[78,79] The isomerization energy barriers for small *N*-methylated model peptide systems (tertiary amides) were calculated with quantum mechanical methods and measured with NMR spectroscopy experiments and was found to be between 12-22 kcal/mol (depending on the solvent system),[43,86] while for secondary amides (non-methylated amides) ranged between 18-22 kcal/mol.[86]

The synthesis of structurally constrained peptides can be achieved with several strategies, the most popular being cyclisation of the peptide.[87] Cyclic peptides can be formed by several approaches, such as head-to-tail, head-to-side chain, side chain-to-tail or side chain-to-side chain, among others.[88] Synthesis of self-assembled peptides, which includes non-covalent and reversible interactions, such as van der Waals, hydrogen bonds, hydrophobic and aromatic π-π stacking, is another way to form secondary structures, such as turns, helices, and sheets of self–assembled material (nanotubes).[89-96]

A γ-turn involves seven atoms in the peptide backbone (**Figure 2.1**) and is characterized by the existence of hydrogen bond interaction between carboxyl group of residue (i) and NH of residue (i + 2).[97] The presence of γ-turns is far less abundant in peptides and proteins than β-turns.[97-99] The profile of this axial or classical γ-turn in Ramachandran plot is defined as $\phi = +75°$, $\psi = -65°$, whereas when $\phi = -75°$ and $\psi = +65°$ it is known as the equatorial or inverse form.[97] A normal H-bond distance (3-5 Å) is required for stabilization of folded peptides containing this turn.[97] Normally, some variation of the ω value ($|\omega| \approx 10°$) is also necessary for formation of the hydrogen bond.[97,100]

One of the most eminent secondary peptide structures is the β-turn, which consists of at least four amino acids,[101] with residue numbers (i), (i + 1), (i + 2), (i + 3) (**Figure 2.1**). A general characteristic of β-turns is the occurrence of a hydrogen bonding interaction between CO group of first amino acid (i) with NH (i + 3).[101-104] There are four parameters that confirm the existence of a

β-turn. First, adequate hydrogen bond interactions, which provide a distance around 7 Å for $d_{critical}$ between atoms Cα(i) and Cα(i + 3).[101,105-107] Second, the virtual torsion angles (τ), defined by atoms Cα(i), Cα(i + 1), Cα(i + 2), Cα(i + 3), should fall between -90° ≤ τ ≤ +90°.[101,105-107] Third, the distances between the carbonyl oxygen of residue (i) and NH of residue (i + 3) should be less than 4 Å.[101,108] When all four criteria are met, the peptide exhibits tight β-turn characteristics.



γ-turn β-turn

Figure 2.1. Criteria used in the identification of γ- and β-turn characteristics[97,100,101]

Proline is known to be an active β-turn inducer when placed in the (i + 1) position in peptide backbones. In an effort to find alternative sources of β-turn inducers, our group has previously reported pentacycloundecane (PCU) and trishomocubane amino acid cage compounds.[101,109,110] Herein we present another new tool to form β-turns by taking advantage of the capability of the *N*-oxide moiety to perform strong hydrogen bond interactions when introduced to proline.

Significant progress in establishing the conformation of peptides and proteins from NMR studies has been achieved from the relationship between chemical shift and structure.[111] In addition, computational methods have been employed as useful tools to supplement these experimental studies, particularly in the absence of X-ray data.[112-115]

O'Neil and co-workers studied the conformational effect of proline *N*-oxides [P(NO)] using either an *N*-alkyl-P(NO)-amide or *N*-alkyl-P(NO) containing dipeptide and showed that hydrogen bonding takes place mainly through a six-member ring (**Figure 2.2**). In addition it was reported that the formation of hydrogen bond between amide protons further away and the *N*-oxide unlikely and if formed, quite weak.[116,117]

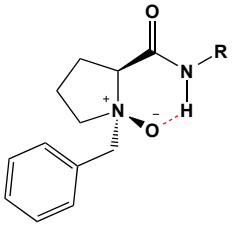


Figure 2.2. Proposed *N*-oxide hydrogen bonding arrangement by O'Neil and co-workers[117]

Since the NO^- oxygen is replacing the normal carbonyl oxygen *as per* the standard turn definition, the observed turn with the *N*-oxide cannot be classified as a normal γ- turn. The number of atoms involved in a classical γ-turn is 7. In this quasi-turn case it consists of 6 atoms.

We decided to expand on this study, by investigating the scope and limitations of a tertiary proline *N*-oxide, combined with selective *N*-methylations as modulators of tetrapeptide conformations through hydrogen bonds with neighbouring amide NH protons.

Two different families of peptides were earmarked. The sequence of the first was chosen based on optimal β-turn characteristics and the second with amino acids known not to induce turns. Several tetrapeptides, which contain *N*-oxide-*N*-benzylproline, as *N*-terminal residue (**Figure 2.3**) were prepared, and their conformational preferences were studied with NMR spectroscopy. Proline followed by glycine is known to be a strong β-turn combination.[29] For the first peptide family we therefore chose [**Bz**(**NO**)**PGNF**]. Asparagine (**N**) and phenylalanine (**F**) are also known to assist with turn formation.[118,119] For the second peptide family we selected [**Bz**(**NO**)**PIVQ**]. Both isoleucine (**I**) and valine (**V**) are known to prevent β-turn formation of short peptides.[118-121] Glutamine for the final residue was chosen since it is bulkier than asparagine and it also has an amide side chain. Control peptides without the *N*-oxide were also synthesised.

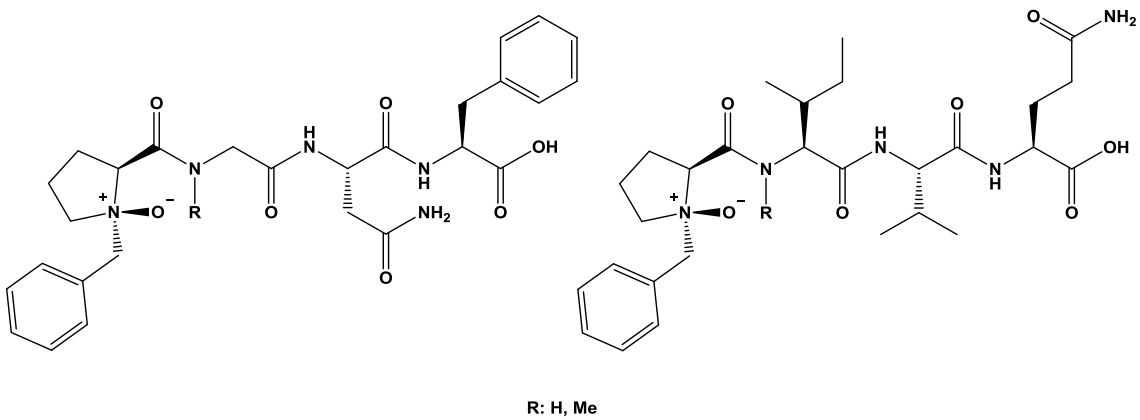
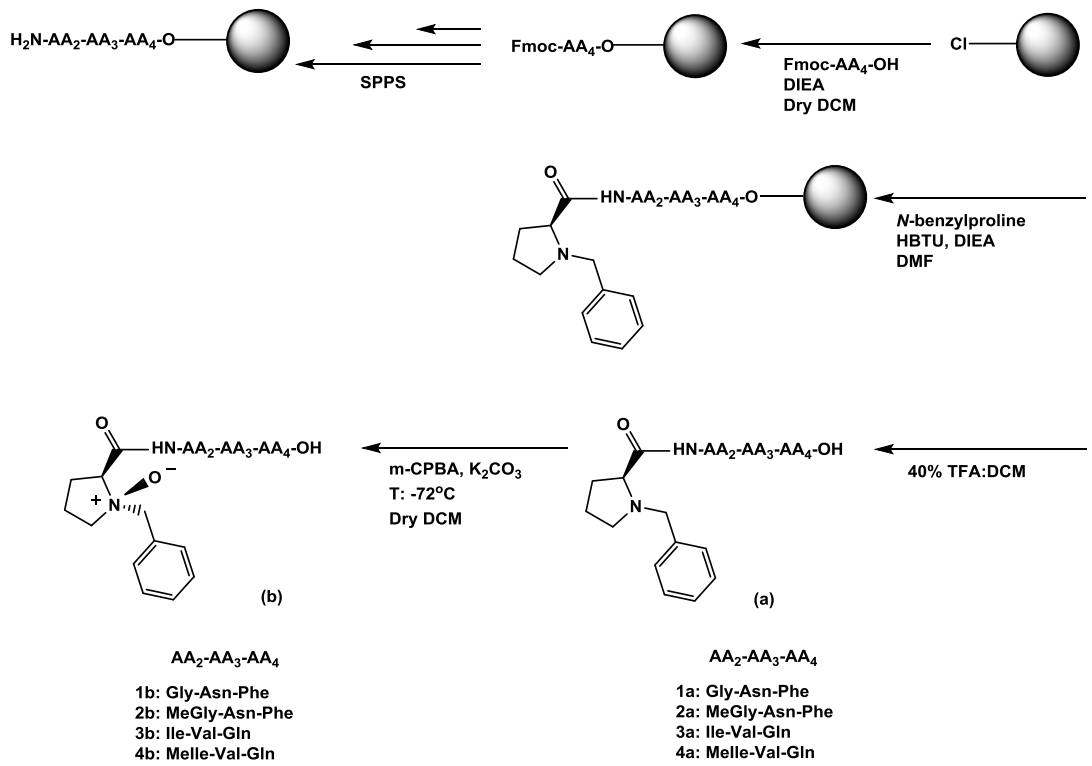


Figure 2.3. *N*-oxide-proline tetrapeptides. [**Bz**(**NO**)**PGNF**], [**Bz**(**NO**)**PMeGNF**], [**Bz**(**NO**)**PIVQ**] and [**Bz**(**NO**)**PMeIVQ**]

In the case of the *N*-oxide and *N*-methylated peptides, potential hydrogen bond interactions between the (i + 2) amide proton and the *N*-oxide group is possible. Since the NO^- oxygen is replacing the normal carbonyl oxygen *as per* the standard β-turn definition (see **Figure 2.1** for the definition), such a turn with the *N*-oxide cannot be classified as a normal β-turn. The number of atoms involved in a classical β-turn is 10. In this quasi-turn case, the number of atoms is 9.

1.2 Results and discussion

Peptides were synthesized *via* a solid-phase approach on 2-chlorotrityl-chloride (CTC)-resin with HBTU/DIEA as a coupling cocktail using Fmoc protection of the amino groups. For better control of the synthetic process, previously prepared *N*-benzylproline was incorporated as the last building block.[122] At the end of the synthesis the peptides were cleaved from the resin with 40% TFA in DCM and the tertiary amine of the proline was selectively oxidized using (*m*-CPBA) and K_2CO_3 in DCM at –72°C (**Scheme 2.1**). Peptides were purified *via* a semi-preparative HPLC with a C_8 column and the structures were characterized using NMR and HRMS.



Scheme 2.1. Synthetic strategy used to prepare the proposed peptides

NMR techniques were employed to identify the hydrogen bond acceptor for NO oxygen of the proline *N*-oxide and determine the conformational preferences and stabilities of these series of molecules.

1.2.1 Comparative structural study on the peptides designed for optimal β-turn characteristics

The four structures for the first family of peptides are presented in **Figure 2.4**.

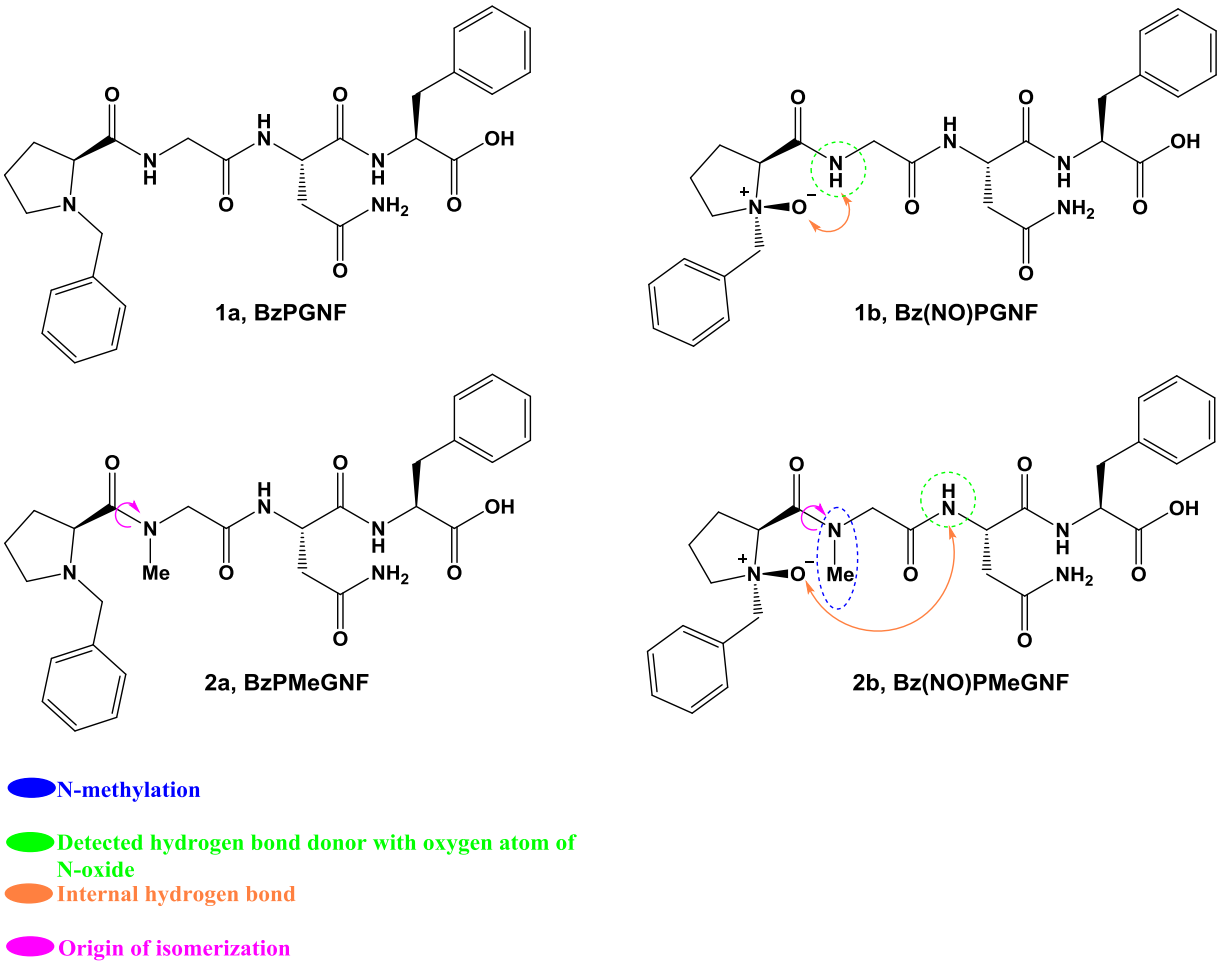


Figure 2.4. Peptides theoretically designed for optimal β-turn characteristics

Complete NMR assignments of all peptides were made utilising 2D NMR techniques. These assignments are presented in the Supplementary Material. Three aspects were monitored: (a) the ratio of *cis/trans* isomerization, (b) deshielding of the amide protons (NH) due to through space deshielding of the NO^- oxygen and (c) NOE interactions characteristic of β-turns.

From the NMR data, it was clear that at least two dominant conformations were present in **2a** and **2b**. These are ascribed to *cis/trans* isomers of the *N*-methylated peptide bonds, as shown in **Figure 2.4**. The *cis/trans* ratios were determined from the integration of distinct proton signals. A summary of these results is presented in **Table 2.1**.

Table 2.1. *Cis/trans* isomerizations of proline amide bonds at the *C*-termini of peptides **1** and **2** at room temperature. Data obtained from proton NMR integrations in DMSO-d_6.

Symbol	Sequence	*cis %*	*trans %*
1a	**BzPGNF**	0	100
1b	**Bz(NO)PGNF**	0	100*
2a	**BzPMeGNF**	47	53
2b	**Bz(NO)PMeGNF**	29	71

* This sequence shows two conformations (See **Table 2.2**).

1.2.1.1 Comparison between compounds 1a and 1b

Peptide **1a** displayed only the *trans*-isomer. After assigning each peak in ^{1}H NMR and 2D NMR, it seemed that this molecule shapes somehow that fits on stable hydrogen bonding pattern.

Peptide **1b** appeared as two conformers (major and minor). ROESY NMR data indicated that both conformers are *trans* with respect to the amide bonds (**Figure 2.5**). The proline α-proton gives an NOE interaction with the (i + 1) NH proton.

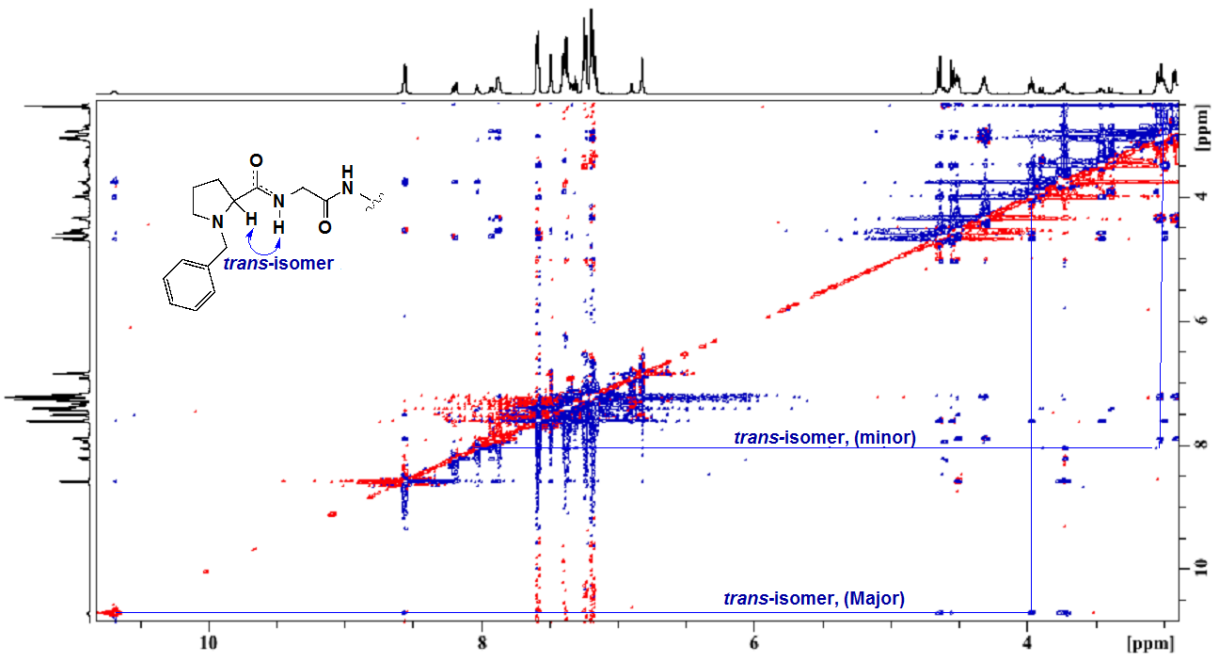


Figure 2.5. Detection of both *trans* conformers by usage of ROESY in 1b, [**Bz(NO)PGNF**] peptide at room temperature. The water peak is suppressed. (Solvent: DMSO-d_6)

It should be noted that the percentage of minor conformation in **1b** was quite small (15%), hence only a weak NOE interaction was observed. An analysis of these two conformations was made from the NMR data and is presented in **Table 2.2**.

Table 2.2. NMR chemical shifts of amide groups in **1a** and **1b**. Chemical shifts are reported in ppm. (Solvent: DMSO-d_6)

Amide	**1a, (BzPGNF)**	**1b, [Bz(NO)PGNF]**	
		major*	**minor***
NH-Gly	7.98	10.78	8.02
NH- Asn	8.23	8.55	8.19
NH-Phe	7.48	7.86	7.92

* Two conformations were observed. The major conformation appeared to exhibit some γ-turn character (NO^- and prominent (i + 1) NH interaction). The minor conformation is possibly a more extended peptide conformation (lack of NO^- and NH (i + 1) interaction).

The ^{1}H NMR data indicates that the first amide (i + 1) of the major isomer of **1b** prefers to engage with the *N*-oxide moiety. This is evident from the downfield shift of the (i + 1) amide proton in the proton NMR spectra.[123] This shift is most possibly the result of intramolecular hydrogen bonding interactions between the amide proton and the *N*-oxide oxygen atom, causing through space deshielding and suggests γ-turn characteristics.[29] On the other hand, this interaction is absent for the minor conformation, suggesting it does not include the quasi γ-turn. The variable temperature NMR data indicated that the major isomer (quasi γ-turn) begins to loose the turn structure and in favour of perhaps a more extended structure upon increasing the temperature. Protein structures are stabilised through intramolecular hydrogen bonding and with increasing temperature this interaction is destroyed in favour of the more extended conformation (**Figure 2.6**).

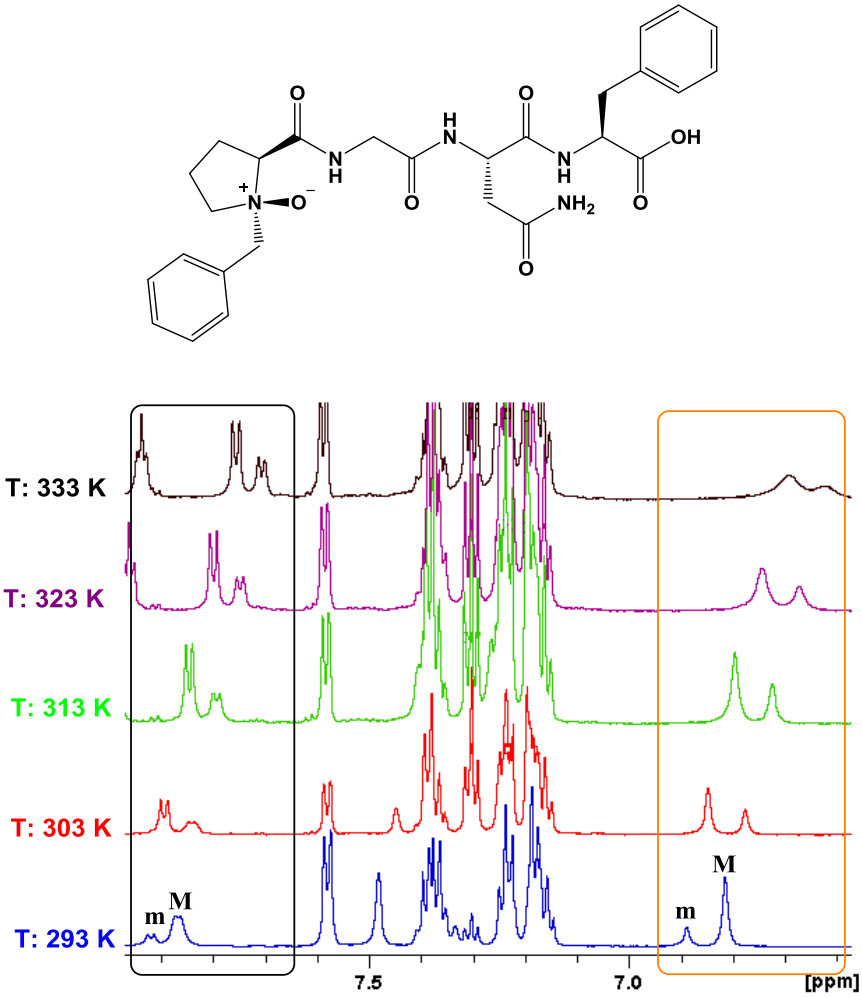


Figure 2.6. **A** thermodynamic study of **1b,** [**Bz(NO)PGNF**]. Chemical shifts are reported in ppm. Major (**M**) product converts to minor (**m**) product with increasing temperature. (Solvent: DMSO-d_6).

1.2.1.2 Comparison between compounds 2a and 2b

Peptide **2a** includes *N*-methylated glycine as the first amino acid following *N*-benzylproline. As expected, both *cis* and *trans*-isomers exist and the *cis/trans* isomerization ratio is 47:53 at room temperature (**Figure 2.7**).

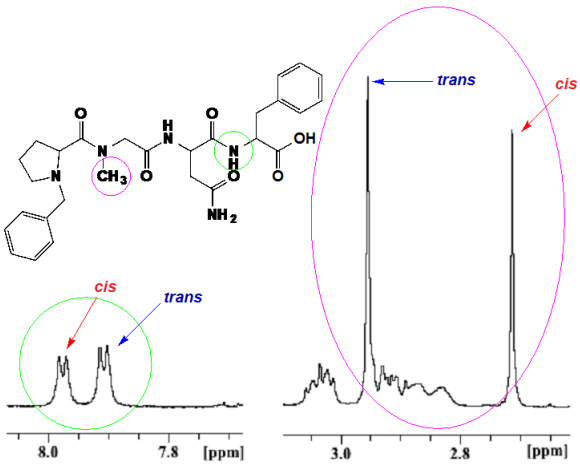


Figure 2.7. Ratio between *cis* and *trans*-isomers in **2a,** (**BzPMeGNF**) (47% *cis*, 53% *trans*) at room temperature. (Solvent: DMSO-d_6)

The isomers were verified from the ROESY data (**Figure 2.8**). It is clear that the *trans*-conformation should experience a through space NOE interaction between the proline Hα and the methyl protons (*N*-Me). This was absent for the *cis*-isomer due to the methyl protons (*N*-Me) pointing in the opposite direction.

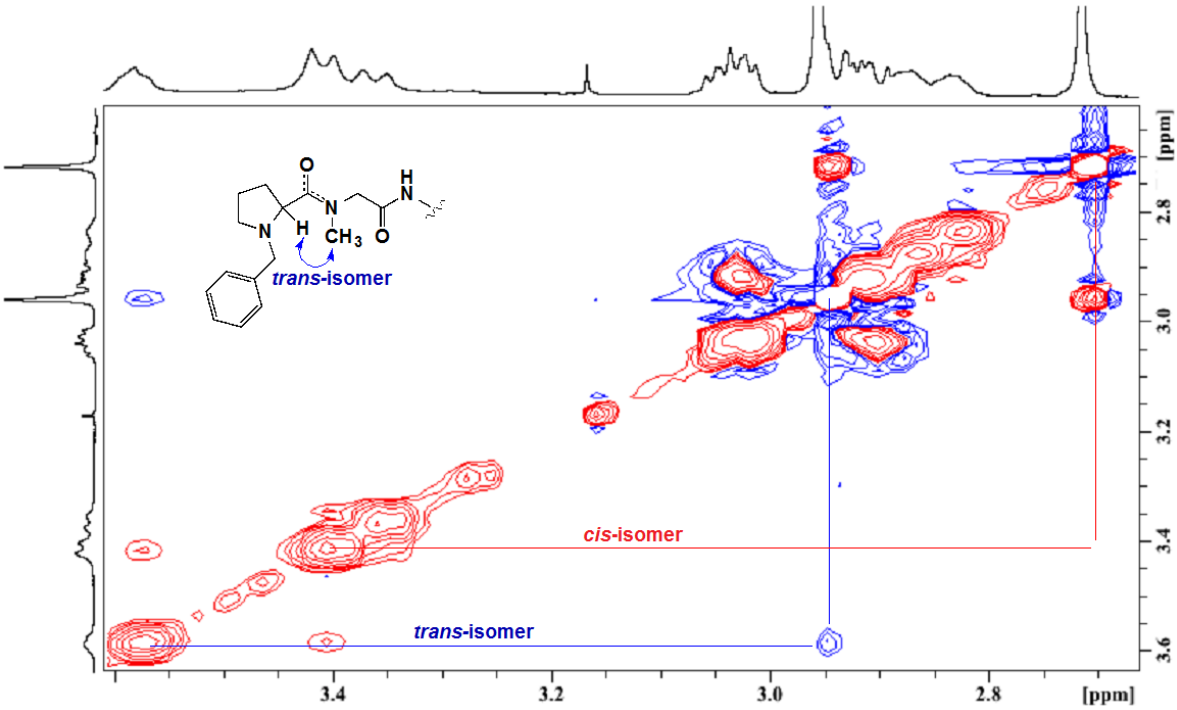


Figure 2.8. ROESY study for the detection of *cis*- and *trans*-isomers in **2a,** (**BzPMeGNF**) at room temperature. The water peak is suppressed. (Solvent: DMSO-d_6)

With regards to **2b**, The complete NMR elucidation of the *cis*-isomer could not be achieved due to a low signal to noise ratio and overlapping of signals with the *trans*-isomer. The *cis*-isomer (29%) was not involved in hydrogen bond interaction (**Figure 2.11**).

For the *trans*-isomer (71%) of **2b**, the backbone amide proton of asparagine (i + 2) was de-shielded (about 9 ppm) due to the close proximity of the *N*-oxide oxygen (**Figures 2.9 and 2.10**) which causes a moderate hydrogen bonding interaction. This result suggests that the *N*-oxide moiety can be used as a β-turn inducer. However, as mentioned before this turn is not a classical β-turn (**Figure 2.1**)[29] but rather a quasi, or perhaps a new type of β-turn. This turn was confirmed by correlations from ROESY NMR **(Table 2.3)**.

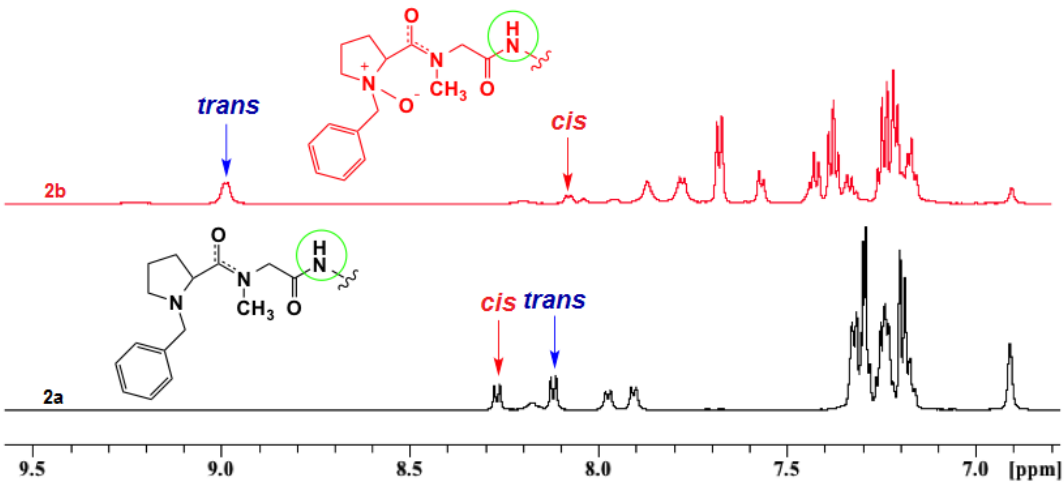


Figure 2.9. NMR proton chemical shifts in the down field region for **2a,** (**BzPMeGNF**) and **2b,** [**Bz**(**NO**)**PMeGNF**] at room temperature. (Solvent: DMSO-d_6)

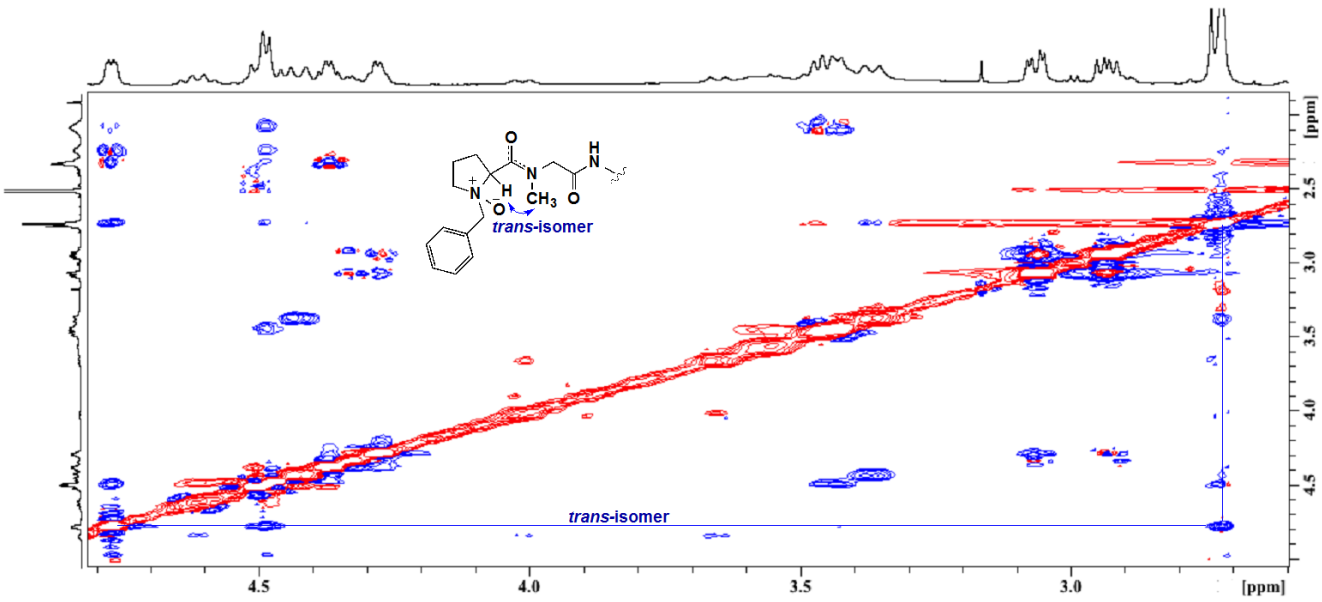


Figure 2.10. ROESY study for detection of *cis*- and *trans*-isomers in **2b,** [**Bz(NO)PMeGNF**] at room temperature. The water peak is suppressed. (Solvent: DMSO-d_6)

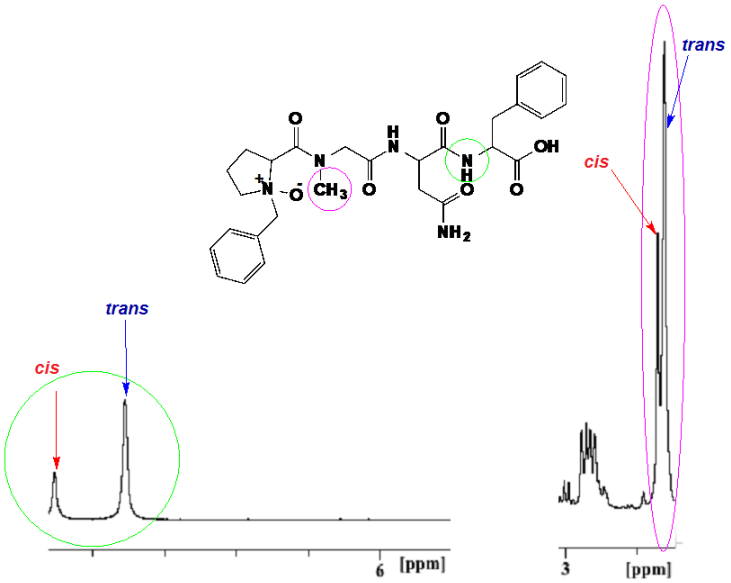


Figure 2.11. Ratio between *cis*- and *trans*-isomers in **2b,** [**Bz(NO)PMeGNF**] (29% *cis*, 71% *trans*) at room temperature. (Solvent: DMSO-d_6)

ROESY data for *trans*-**2b** are presented in **Table 2.3**. The correlations between H-21 with H-11, H-14 and H-15 as well as the correlations between H-26 and H-20, H-17 provide convincing proof for the presence of a quasi β-turn in **2b**. These correlations clearly display that the intramolecular

hydrogen bonding interaction between NO^- and backbone asparagine NH is able to induce folding of the peptide (**Table 2.3**).

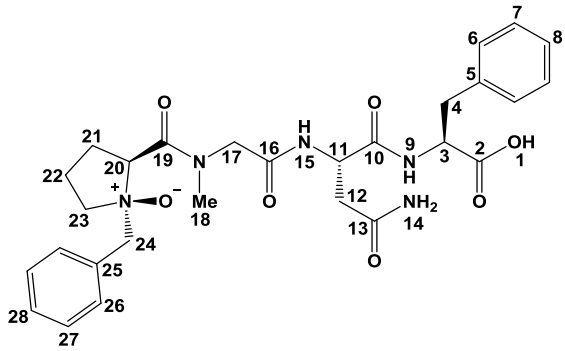


Table 2.3. NOE correlations for *trans*-**2b** at room temperature in DMSO-d_6, [**Bz**(**NO**)**PMeGNF**]

Atom Number	Correlated hydrogen atoms
18	15
21	11, 14, 15, 26
22	17
26	17,20

1.2.1.3 Comparison between compounds 1b and 2b

Due to the flexible nature of glycine in the peptide backbone and the absence of a chiral centre, the hydrogen bond interaction between the *N*-oxide oxygen and the amide proton appears to be less prominent in **1b**. When the first hydrogen bond donor in position (i + 1) is blocked with a methyl group as in the case of **2b**, the possibility of hydrogen bonding between the *N*-oxide moiety and this amide group (i + 1) is eliminated. The shielding effect of the NO^- on the corresponding amide NH protons can be seen in **Figure 2.12**. For **1b**, the hydrogen bond interaction between the amide proton of glycine (i + 1) and the negative NO^- oxygen at the *N*-terminal of proline provides a stronger deshielding interaction, compared to the amide proton of the more distant asparagine (i + 2) and NO^- of **2b**.

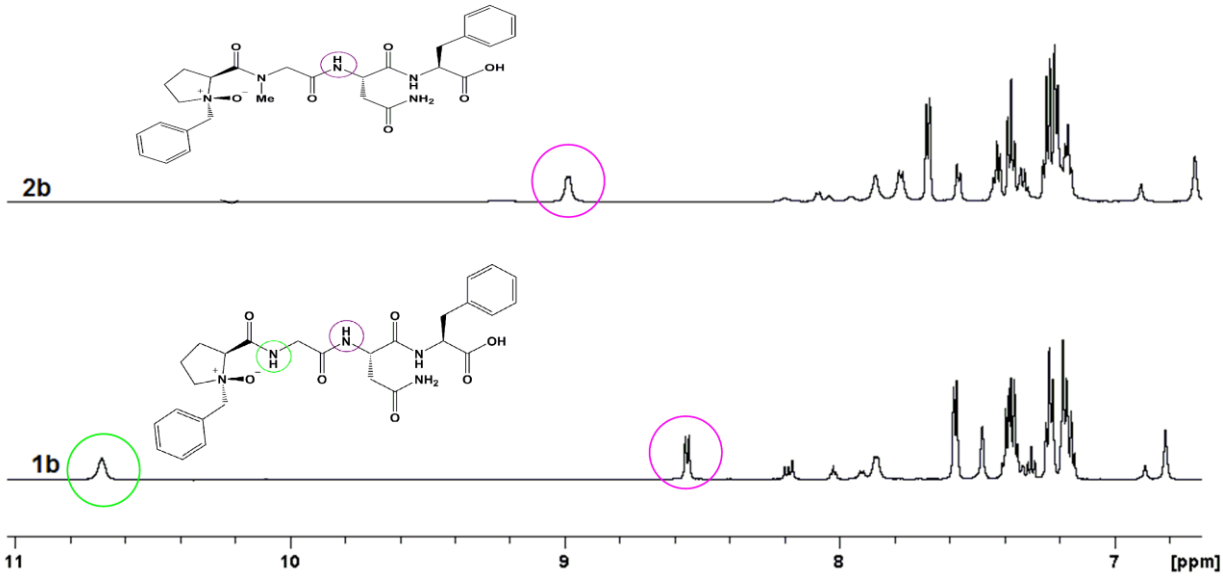


Figure 2.12. Estimation of the hydrogen bonding potential in **1b**, [**Bz**(**NO**)**PGNF**] and **2b**, [**Bz**(**NO**)**PMeGNF**] based on NMR chemical shifts at room temperature (Solvent: DMSO-d_6)

1.2.2 Comparative structural study on peptides designed for minimal β-turn characteristics

The four structures for the second family of peptides are presented in **Figure 2.13**. Complete NMR assignments for all peptides were made utilising 2D NMR techniques. These assignments are presented in the Supplementary Material.

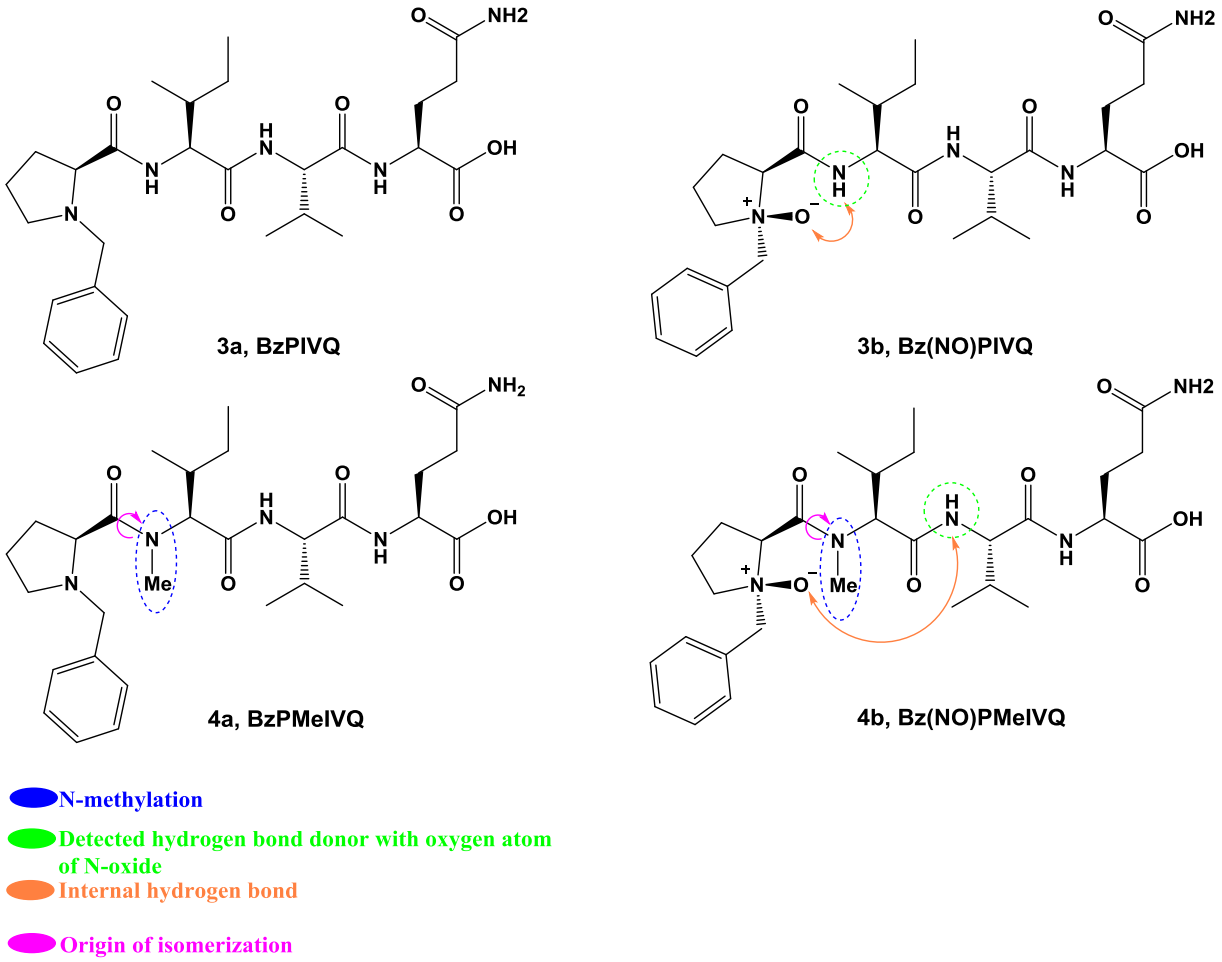


Figure 2.13. Peptides designed for minimal β-turn characteristics

The peptides structures were studied using 1D and 2D NMR techniques, and full elucidation of each peptide was achieved. Three aspects were again monitored: (a) the ratio of *cis/trans* isomerization and (b) deshielding of the amide NH protons due to through space deshielding of the NO^- oxygen and (c) prominent NOE interactions. As expected, only *trans* amide conformations were observed for the normal peptides **3a** and **3b**. It was clear that at least two conformations were presented for **4a** and **4b**, this being ascribed to *cis/trans*-isomers of the *N*-methylated peptide bonds, as illustrated in

Figure 2.13. The *cis/trans* ratios were determined from the integration of distinct proton signals. A summary of these results is presented in **Table 2.4**.

Table 2.4. *Cis/trans* isomers of the proline amide bonds at the *C*-termini at room temperature. Data obtained from proton NMR integrations are in DMSO-*d*6.

Symbol	Sequence	*cis* %	*trans* %
3a	**BzPIVQ**	0	100
3b	**Bz(NO)PIVQ**	0	100
4a	**BzPMeIVQ**	40	60
4b	**Bz(NO)PMeIVQ**	77	23

1.2.2.1 Comparison between 3a and 3b

Comparisons of the NMR chemical shifts for NH in the (i + 1) positions were made. A four-ppm difference between the isoleucine amide proton signals (**Figure 2.14, 3a** appears at 7.85 ppm and **3b** at 12.05 ppm) is evidence for the presence of an intramolecular hydrogen bonding interaction for **3b**. This through space deshielding effect was clearly an indication of a tight hydrogen bond interaction between the NH (i + 1) proton and the negatively charged NO^- oxygen atom. These results are in agreement with the reports by O'Neil *et al.*[116,117] This is a quasi γ-turn. No extraordinary ROESY correlations between proline and the rest of the peptide were noticed in either case.

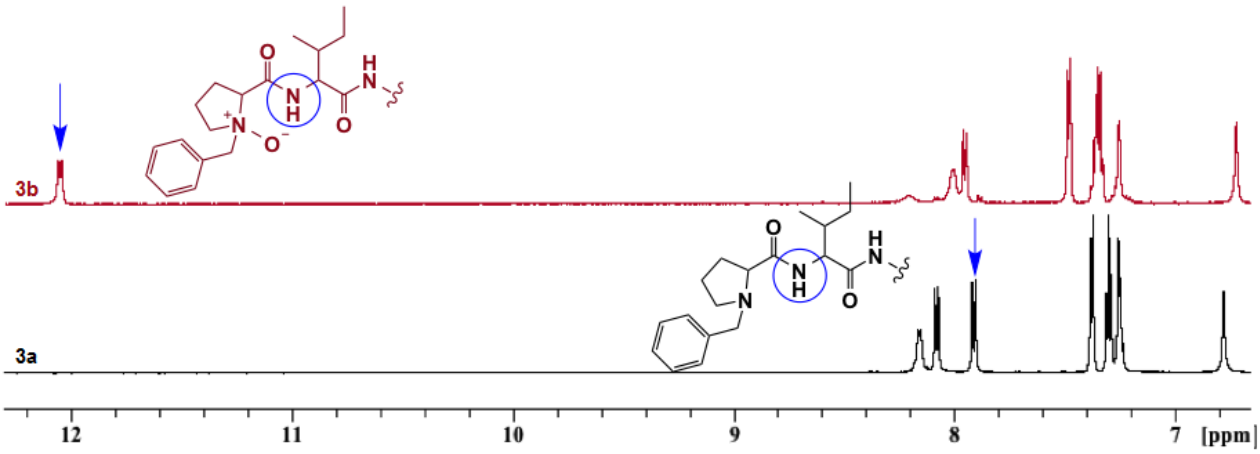


Figure 2.14. NMR proton chemical shifts in the down field region for **3a**, (**BzPIVQ**) and **3b,** [**Bz(NO)PIVQ]** at room temperature (Solvent: DMSO-d_6)

In order to analyse the versatile nature of this NH to NO^- hydrogen bonding interaction, the *N*-methylation of the isoleucine amide nitrogen (i + 1) was investigated.

1.2.2.2 Comparison between 4a and 4b

From **Table 2.4**, it is clear that *N*-methylation induces *cis*- and *trans*-isomers for both peptides (**4a** and **4b**). In **4a**, the *cis*-isomer is 40% and the *trans*-isomer 60% according to the proton spectrum at ambient temperature (**Figure 2.15**). Note that the *cis*-isomer is favoured by the polar NMR solvent (DMSO-d_6).

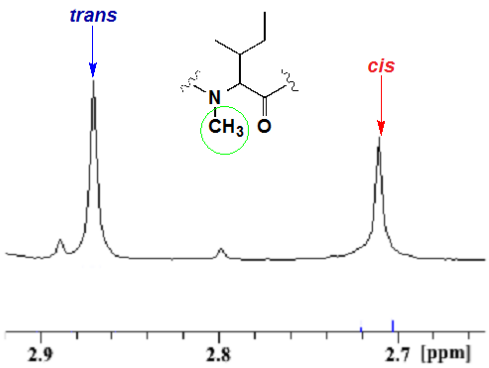


Figure 2.15. Ratio between *cis*- and *trans*-isomers in **4a**, (**BzPMeIVQ**), (40% *cis*, 60% *trans*) at room temperature. (Solvent: DMSO-d_6)

Both isomers were observed in a ROESY NMR experiment, as is shown in **Figure 2.16.**

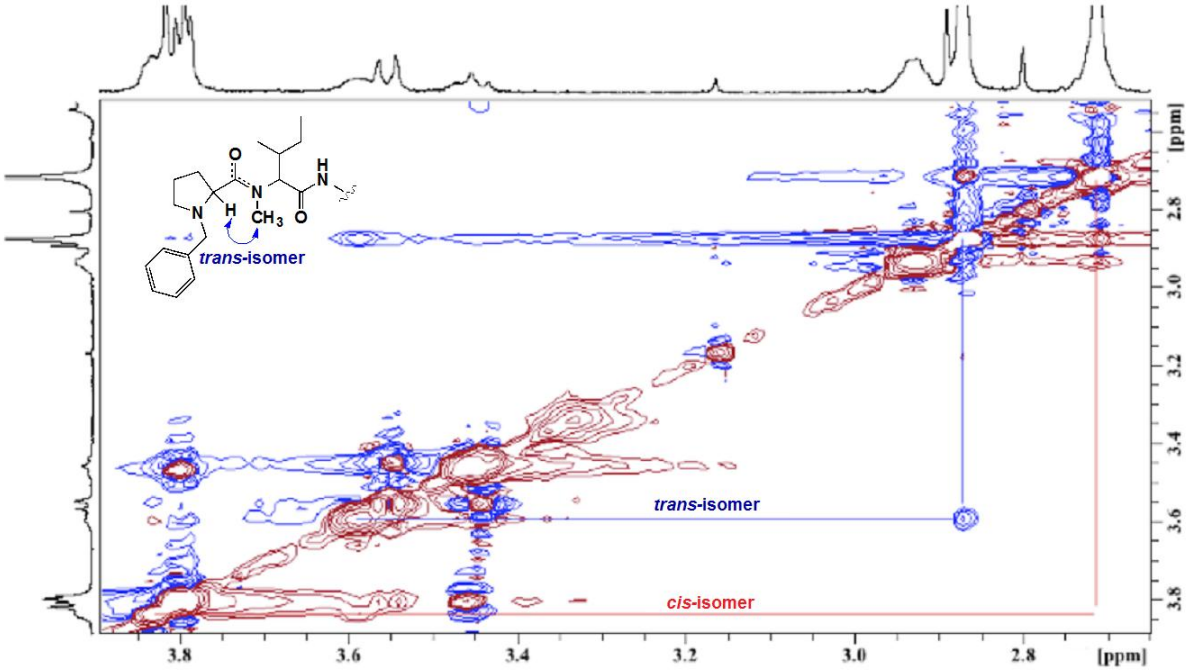


Figure 2.16. ROESY study for detection of *cis*- and *trans*-isomers in **4a**, (**BzPMeIVQ**) at room temperature. The water peak is suppressed (Solvent: DMSO-d_6)

Further investigations were conducted to observe if any folding occurred in **4a**. No extra-ordinary ROESY correlations were observed, which indicates that the distance between protons of two different residues in the same backbone is more than 5 Å or the system is too flexible to observe NOE interactions. This evidence suggests that the backbone does not form any stable turn or fold.

Similarly, the NMR of **4b** presented multiple isomers. The ratio could not be accurately established from the *N*-Me protons, due to overlap. The glutamic acid side chain amide protons revealed a 77:23 ratio in favour of the *cis*-isomer (**Figure 2.17**). Confirmation of the isomer assignments was achieved when it was established that the NH proton of valine (i + 2) at about 9.9 ppm experienced through space deshielding due to intramolecular hydrogen bonding with the *N*-oxide moiety (**Figure 2.18**). Further evidence for this elucidation was obtained from the ROESY spectra of **4b** (**Figure 2.19**).

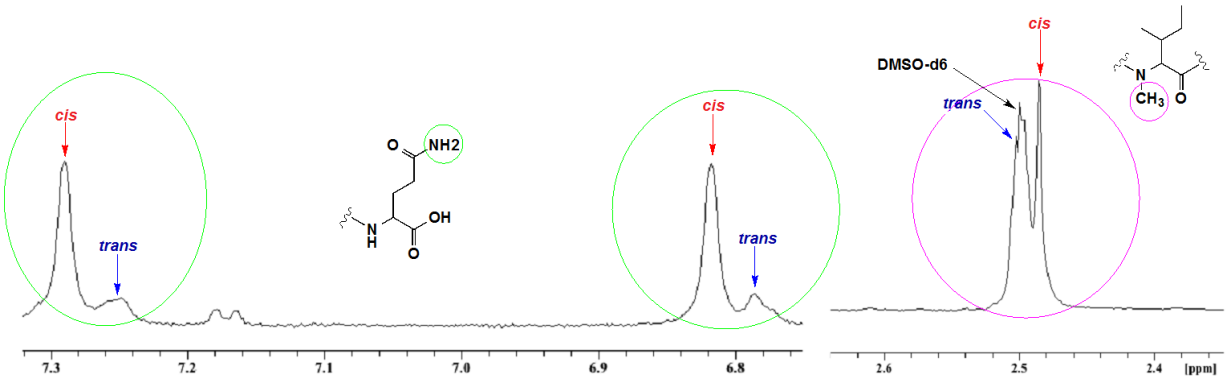


Figure 2.17. Ratio between *cis*- and *trans*-isomers in **4b**, [**Bz(NO)PMeIVQ**] (77% *cis*, 23% *trans*) at room temperature. (Solvent: DMSO-d_6)

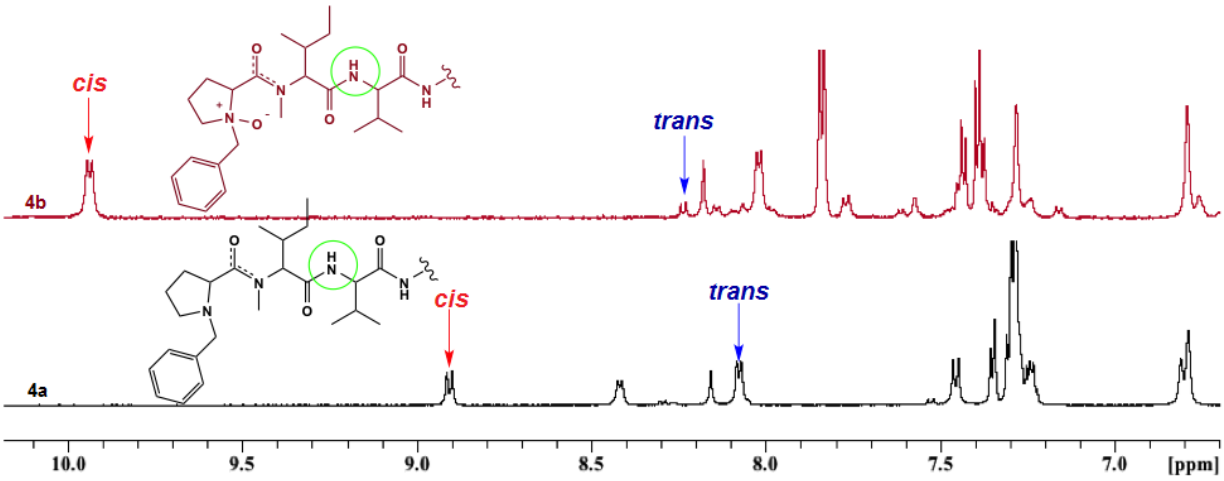


Figure 2.18. NMR proton chemical shifts in the down field region for **4a**, (**BzPMeIVQ**) and **4b**, [**Bz**(**NO**)**PMeIVQ**] at room temperature. (Solvent: DMSO-d_6)

We initially chose isoleucine and valine because they have been reported to prevent β-turns.[118-121] This turn (*cis*-**4b**) appears to be a quasi β-turn, created by the *N*-oxide moiety, in the presence of these residues. To our knowledge this is the first example of such a secondary structure.

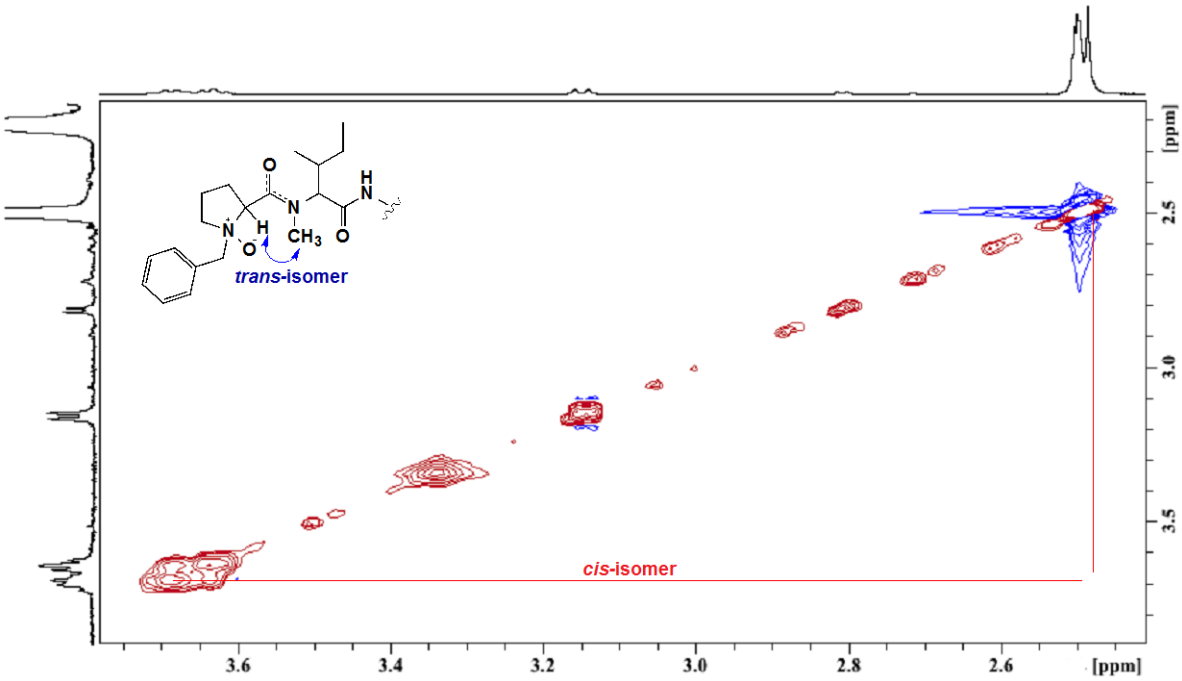


Figure 2.19. ROESY studies for the detection of *cis*- and *trans*-isomers in **4b,** [**Bz-P**(**NO**)**-MeI-V-Q**]. The absence of a NOE correlation between the *cis*-*N*-methyl group and the proline Hα, assisted to differentiate between the conformations. The water peak is suppressed. The spectra was obtained at room temperature. (Solvent: DMSO-d_6)

Development of a quasi β-turn secondary structure (see Figure 2.13) in the presence of bulky amino acids, such as isoleucine and valine, is therefore a novel and unique achievement, and appears to only occur as result of the P(NO) in the peptide backbone.

The β-turn character of *cis*-**4b** was confirmed with several long-range correlations from ROESY NMR, as demonstrated in **Table 2.5** (the ROESY spectra including correlations are available in Supplementary Material). Convincing evidences for the presence of a quasi β-turn in **4b** are: (a) correlations between H-20 with H-12, H-17 and H-18, (b) a correlation between H-23 with H-12 and (c) relation between H-24 and H-12. (d) Existence of NOE correlations between H-26 and H-15, H-

17 and H-18 (e) a correlation between H-28 and H-12. These correlations support the presence of intramolecular hydrogen bonding interactions between NO^- and the backbone asparagine NH, induced folding of the peptide.

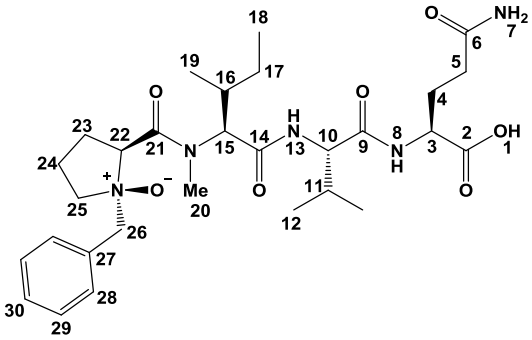


Table 2.5. NOE correlations of *cis*-**4b**, [**Bz**(**NO**)**PMeIVQ**], at room temperature in DMSO-d_6

Atom Number	Correlated hydrogen atoms
20	12, ,16, 17, 18
24	12
26	15
28	13

1.2.2.3 Comparison between 3b and 4b

The potential of the *N*-oxide group to create hydrogen bonding can be clearly seen in **3b** and **4b**. The amide proton of isoleucine (i + 1) in **3b** appeared at about 12 ppm, whereas the amide of valine (i + 3), registered at approximately 10 ppm (**Figure 2.20**). These results indicate that the distance between the hydrogen bond donor and acceptor (*N*-oxide) plays an important role in peptides with sterically hindered amino acids. In this case it forms a very strong hydrogen bond between NO^- and the closest amide as was evident from the large deshielding effect on the (i + 1) amide proton in **3b** (**Figure 2.20**). The *trans*-conformation of **3b** and the steric hindrance imposed by isoleucine and valine appeared to prevent any interaction between the other amide protons and the NO^-. In contrast, the *N*-oxide moiety in **4b** revealed a 77% *cis*-conformation which was able to overcome this difficulty to hydrogen bond between NH (i + 2) and NO^-.

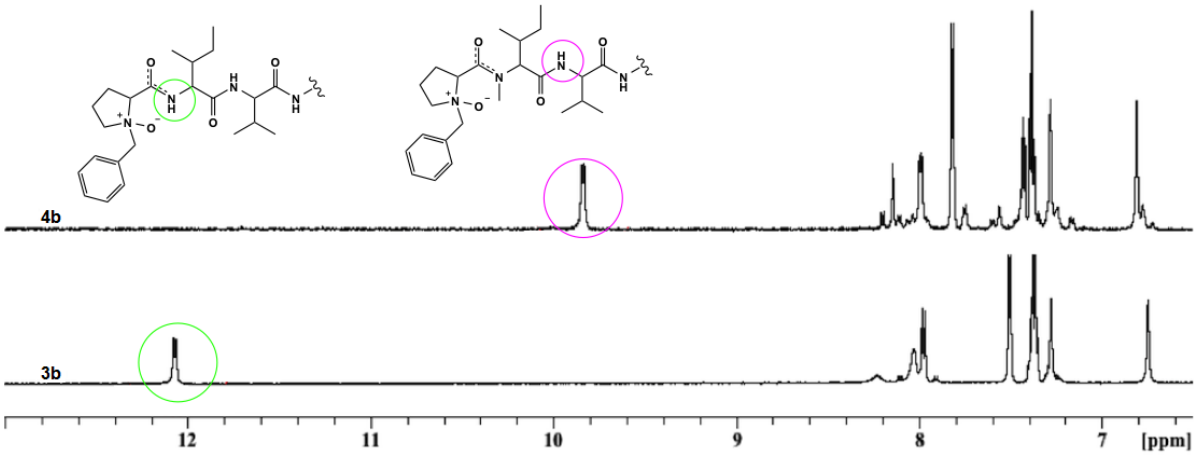


Figure 2.20. Estimation of hydrogen bond potential, based on NMR shift in **3b**, [**Bz**(**NO**)**PIVQ**] and **4b**, [**Bz**(**NO**)**PMeIVQ**] at room temperature (Solvent: DMSO-d_6)

1.2.3 Thermal coefficient NMR investigation: Nature of the hydrogen bond interaction between the proline N-oxide and the backbone NH protons.

As discussed before, the presence of this hydrogen bond could be detected by observing the chemical shifts of backbone amide protons in the ^{1}H NMR spectra. Furthermore, analyses of the thermal behaviour of hydrogen bonds in the peptides utilizing NMR are an elegant technique to study the exact nature of this interaction.[124-129] Normally, the intramolecular hydrogen bonding interactions between a hydrogen bond donor (amide proton) and the acceptor (carbonyl oxygen) in a peptide is disturbed at higher temperatures; the amide proton signals also experience a downfield shift (less through space deshielding) in the ^{1}H NMR spectra in aprotic solvent systems.[124-129] In aprotic solvents such as DMSO-d_6, when $-\Delta\delta/\Delta T > 5$[1] ppb/K, the typical intramolecular peptide hydrogen bond is absent if the amide protons are solvent-exposed. When $-\Delta\delta/\Delta T < 3$ ppb/K the amide proton is shielded from the solvent, due to hydrogen bonding with any carbonyl oxygen atom.[124-129]

The plotted $-\Delta\delta/\Delta T$ graphs (available with the Supplementary Material) for peptides and *N*-methylated peptides in DMSO-d_6 confirmed that no intramolecular hydrogen bonds exist for peptides **1a–4a**. The presence of hydrogen bonds between the NO^- moiety and hydrogen-bonded NH in different *N*-oxide peptides **1b – 4b** were also investigated with this method. These molecules present a low $-\Delta\delta/\Delta T$ value (-2.64 to 0 ppb/K) for amide protons involving hydrogen bonding with the *N*-oxide moiety. When these amide protons are not involved in hydrogen bonding interactions, the values are > 4 (ppb/K). The results for peptides **1b** – **4b** are presented in in **Figures 2.21, 2.22, 2.23** and **2.24**. The negative value for $-\Delta\delta/\Delta T$ (which renders positive slopes) called for a more in depth analysis.

Langner and Zundel[130] reported that the acid-base equilibria (**AH---B$^-$ ⇔ A$^-$---HB**) plays a very important role in the proton transfer process. Proton transfer systems are characterized by the following: i) All systems of this family possess the same hydrogen bond donor (**AH**) and the acceptors (**B**) are also similar molecules, the only difference is between the pK_a of **B**. ii) Or the acceptor (**B**) is always the same and the donors (**AH**) are similar molecules and the only differences are between their pK_a values.

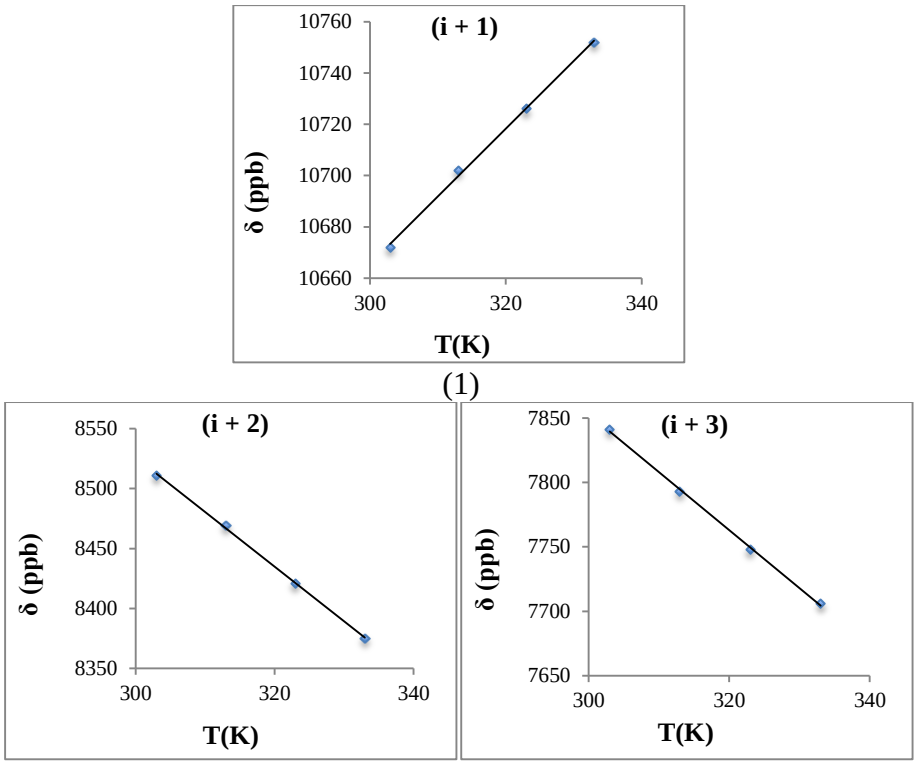
[1] The slopes of these graphs for normal peptides are usually negative.

In such systems, ΔpK_a is defined as the [pK_a **B** - pK_a **AH**].[131,132] Langner and Zundel[130] have explored the interaction between a strong acid (**AH**) such as dimethylphosphinic acid (DMP) and methanesulfonic acid (MSA) with different derivatives of *N*-oxide molecules as the base. It was found that for systems with an increasing ΔpK_a, the proton from the hydrogen bond donor moves closer to the acceptor, which causes deshielding of the mentioned proton. For example, in the case of MSA (ΔpK_a = 2.5) the proton resides with MSA, while for DMP ($\Delta pK_a \approx 6.5$), the proton is shifted completely to the hydrogen bond acceptor and the signal appears in the up field region of the ^{1}H NMR spectra. These results are also consistent with an entropy (*S*) argument since with increasing ΔpK_a, the structures possess more polarity, and hence, a well-ordered environment can be achieved.[130] Therefore, shifting the proton in the direction of the acceptor is due to increasing the amount of the negative entropy.

The hydrogen bond donor (**AH**) in this study was hydrogen-bonded amides and the acceptor (**B**) role was played by *N*-oxide moiety. The proton chemical shift for several of these amides appeared in a region that is known for acid functional groups and this gives strong evidence for the acidic character of such amides. The ΔpK_a value for this system (amide and *N*-oxide) is expected to be low. It is important to note that ΔpK_a is directly related to temperature according to the Van't Hoff and Helmholtz equations.

With this background in mind, the amide proton NMR shifts were measured at different temperatures in order to determine the hydrogen bond character between that and the *N*-oxide oxygen atom.

For *trans*-**1b**, [**Bz**(**NO**)**PGNF**], the $-\Delta\delta/\Delta T$ graphs are presented in **Figure 2.21**. The hydrogen bond interaction between the amide proton (i + 1) and the *N*-oxide oxygen renders a positive slope (gradient = 2.64). This result is typical of normal acid-base equilibria behaviour.[130] The slope of this gradient suggests that the proton transfer in this system was temperature sensitive and the down field proton shift (≈10 ppm) suggested that the amide proton had probably started to transfer to the donor atom (*N*-oxide oxygen). There are two opposing factors playing a role in this case. First, the ΔpK_a value is directly proportional to the temperature and this causes the amide proton (i + 1) to be transferred to the *N*-oxide oxygen atom. Second, the glycine residue possesses less steric hindrance and causes greater flexibility of the peptide at higher temperatures. This flexible nature makes transfer of the amide proton more difficult/less possible.



(1) (2) (3)

(1) HN-Gly (-Δδ/ΔT= -2.64 ppb/K)[2], (2) HN-Asn (-Δδ/ΔT= 4.56 ppb/K), (3) HN-Phe (-Δδ/ΔT= 4.50 3ppb/K) (R^2 > 0.997 for all graphs)

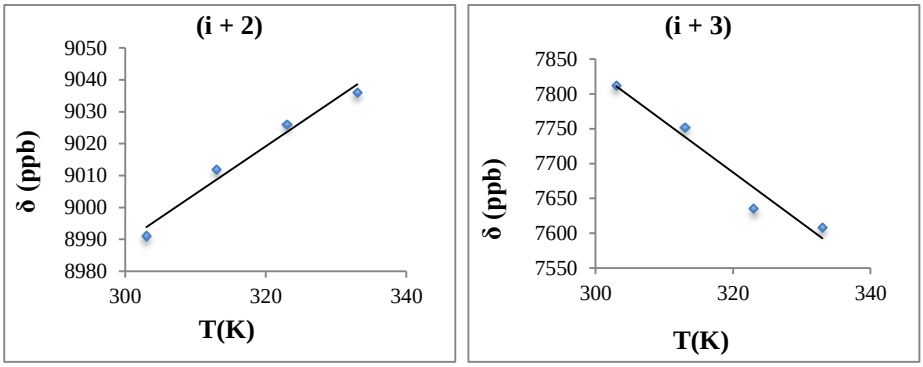
Figure 2.21. The hydrogen bond investigation with thermal coefficient plots for each amide protons of *trans*-**1b**, [**BzP(NO)GNF**]. (Solvent: DMSO-d_6), (temperature: 293-333 K)

The non-hydrogen-bonded amides (i + 2 and i + 3) seemed to be slightly more shielded at higher temperatures with the expected negative slopes (4.56 and 4.50). These values are typical for peptide amide protons in the absence of hydrogen bond interactions.[124-129]

The -Δδ/ΔT plots for both of the backbone amides of *trans*-**2b**, [**Bz(NO)PMeGNF**] are presented in **Figure 2.22**. The hydrogen bond interaction between the amide proton (i + 2) and the *N*-oxide oxygen gave a positive slope (gradient = 1.49). This value verified that the hydrogen bond was not as strong as for **1b** (i + 1). The observed gradient for the (i + 1) system in **1b** was higher (gradient = 2.64) than for the (i + 2) system in **2b** (gradient = 1.49); the latter exhibited less temperature

[2] Unusual slope of the HN-Gly plot is related to an acid-base equilibrium between the amide proton and the *N*-oxide moiety

dependence, most possibly due to the more flexible nature of the larger turn structure. An important difference between the (i + 2) data for **1b** and **2b** was that the sign of the slopes indicative of substantial hydrogen bonding for **2b**. Blocking of the (i + 1) amide proton through *N*-methylation, therefore induces stronger hydrogen bond interaction between the *N*-oxide oxygen atom and the (i + 2) amide proton.



(1) (2)

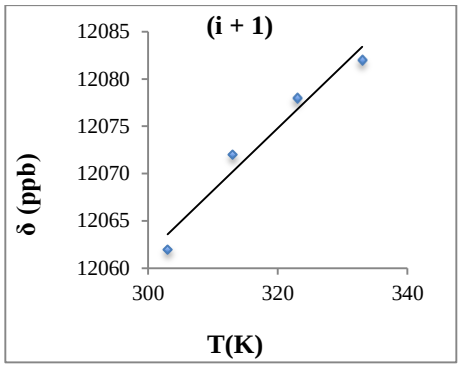
(1) HN-Asn (-Δδ/ΔT= -1.49 ppb/K)[3], (2) HN-Phe (-Δδ/ΔT= 7.29 ppb/K) (R^2 > 0.951 for all graphs)

Figure 2.22. The hydrogen bond investigation with thermal Coefficient plots for each amide protons of *trans*-**2b**, [**Bz**(**NO**)**PMeGNF**]. (Solvent: DMSO-d_6), (temperature: 293-333 K)

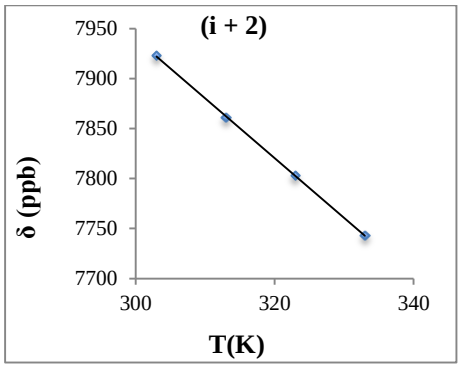
For the next amide (i + 3) of **2b**, this slope was negative (-4.56 ppb/K) suggesting that hydrogen bonding also occurred between corresponding amide proton and the NO^-.

The -Δδ/ΔT plots for the backbone amides of *trans*-**3b**, [**Bz**(**NO**)**PIVQ**] are presented in **Figure 2.23**. The hydrogen bond interaction between the amide proton (i + 1) and the *N*-oxide oxygen resulted in a positive slope (gradient = 0.66). This small value combined with the deshielding experienced by this proton (registered at about 12 ppm) suggested that the proton was being completely transferred from the donor to the acceptor. In comparison to **1b**, the slope for the (i + 1) system of **3b** was much shallower. A potential reason for that was the more rigid nature of peptide **3b**, allowing for substantial transfer of the amide proton to NO^-, even at higher temperatures. It therefore becomes an entropy argument[130] where the corresponding system in **3b** was more ordered.

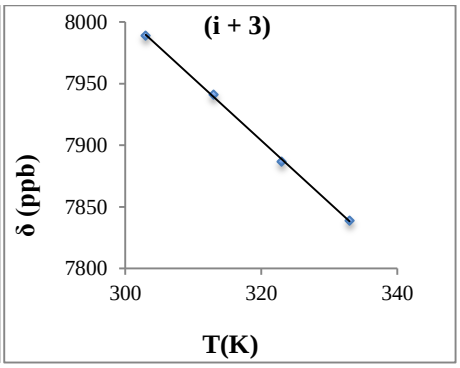
[3] Unusual slope of the HN-Asn plot is related to an acid-base equilibrium between the amide proton and the *N*-oxide moiety.



(1)



(2)



(3)

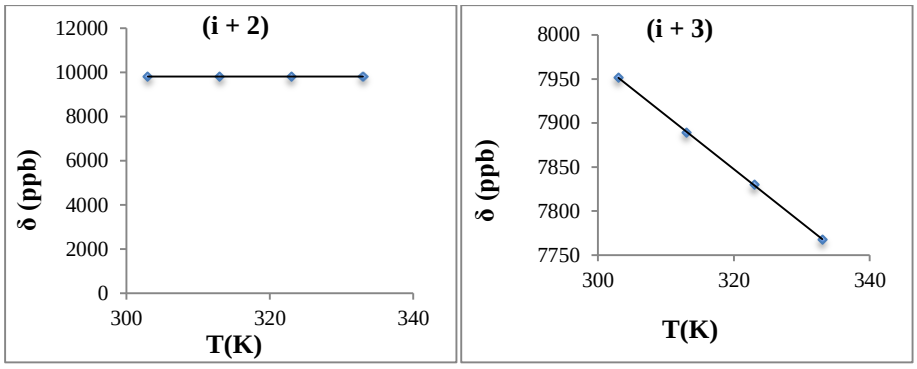
(1) HN-Ile (-Δδ/ΔT= -0.66 ppb/K)[4] (2) HN-Val (-Δδ/ΔT= 5.98 ppb/K), (3) HN-Gln (-Δδ/ΔT= 5.04 ppb/K) (R^2 > 0.959 for all graphs)

Figure 2.23. The hydrogen bond investigation with thermal Coefficient plots for each amide of *trans*-**3b**, [**Bz**(**NO**)**PIVQ**]. (Solvent: DMSO-d_6), (temperature: 293-333 K)

The sign and values of the slopes of *trans*-**3b** for (i + 2) and (i + 3) clearly showed that these backbone amide protons were not involved in hydrogen bonding interactions.

The case of **4b** turned out to be unusual in terms of the behaviour observed so far. The proton NMR spectrum for *trans*-**4b** exhibited no unusual deshielding of any of the amide protons (**Figure 2.24**). On the other hand, the *cis*-isomer displayed an amide proton at about 10 ppm. The -Δδ/ΔT plots for *cis*-**4b**, [Bz P(NO) MeI V Q] illustrated hydrogen bonding interaction between the NH (i + 2) and the *N*-oxide oxygen, but it was not as strong as that observed for the corresponding system in **3b**. The slope of this system is zero, indicating a strong hydrogen bond, but in the absence of acid-base equilibrium.

[4] *Unusual zero slope of the HN-Ile plot is related to hydrogen bond in absence of acid-base equilibrium between the *N*-oxide moiety the amide proton and.



(1) (2)

(1) HN-Val (-Δδ/ΔT= 0 ppb/K), (2) HN-Gln (-Δδ/ΔT= 6.11 ppb/K) (R^2 > 0.999 for all graphs)

Figure 2.24. The hydrogen bond investigation with thermal Coefficient plots for each amide of *cis*-**4b**, [**Bz**(**NO**)**PMeIVQ**]. (Solvent: DMSO-d_6), (temperature: 293-333 K)

The sign and value of the slope of *cis*-**4b** for (i + 3) indicated that the proton did not experience hydrogen bond interactions, similar to the non-hydrogen bonded amides presented before.

The presence of sterically hindered amino acids normally prevents the formation of hydrogen bonding interactions for weak acceptors such as carbonyl groups. However, it can be concluded that for a strong acceptor like *N*-oxide oxygen when combined with *N*-methylation of the (i + 1) amide proton, the existence of bulky side chains in amino acids could still induce stronger hydrogen bond interaction for the (i + 2) system. The possibility of more prominent *cis*-conformations is also possible.

1.3 Experimental

1.3.1 Materials and Methods.

Fmoc-protected amino acids, CTC resin and HBTU were bought from GL-Biochem (Shanghai, China). HPLC–grade CH_3CN, MeOH and peptide synthesis-grade DMF, CH_2Cl_2, DIEA, TFA and all other reagents were purchased from Sigma-Aldrich (Germany). Analytical reversed-phase HPLC was performed on a C_{18} column (4.6 × 150 mm, 5 µm, YMC Triart, UK) with a LC-MS 2020A system (Shimadzu, Kyoto, Japan). Solvents A and B were 0.01% (v/v) formic acid in milli q water, and CH_3CN, respectively. Elution was achieved with linear 0-90% gradients of solvent B to A over 15 minutes at 1 ml/min flow rate, with UV detection at 200 nm. Preparative HPLC was performed with a C_8 column (150 × 21.2 mm, 10 µm, Hichrom) attached to a Shimadzu LC-8A instrument.

Solvents A and B were 0.1% formic acid (v/v) in water and MeOH, respectively and a linear gradient of solvent B to A over 40 minutes, at 15 mL/min flow rate was applied. The UV detection wavelength was set at 200 nm. Purified fractions (>95%) by HPLC were pooled and lyophilized. Purified peptides and conjugates were characterized with HRMS, on Bruker micrOTOF-Q II instrument operating at ambient temperatures and sample 1 ppm. DMSO-d6 was used as a solvent for sample preparation of NMR. H^1 NMR, C^{13} NMR, COSY, HSQC, HMBC and ROESY experiments were performed at ambient temperature on AVANCE III 600 MHz NMR (Bruker, Germany). To achieve better resolution in the NMR spectra, the water peak was suppressed in necessary cases.

1.3.2 General procedure for the synthesis of the tetrapeptides.

Peptides were manually synthesized on a 0.1 mmol scale on CTC resin (0.6 mmol/g). The resin was activated using 10% thionylchloride (v/v) in dry DCM, after which incorporation of the *C*-terminal amino acids was performed with a minimum of four equivalents of Fmoc-amino acid and six equivalents of DIEA in dry DCM for 3 hrs. Elongation of the peptide chains was achieved following standard Fmoc protocols. The coupling conditions were: 5-fold molar excess of Fmoc-amino acid and HBTU, double molar excess of DIEA in DMF. Deprotections were achieved with 20% piperidine in DMF (3 x 10 min). Coupling of *N*-benzylproline was achieved employing the same method. After chain assembly, total deprotection and cleavage was carried out with TFA/DCM (40:60) for one hour and the peptides were precipitated by adding chilled diethyl ether. After evaporation to dryness, the solid forms of the crude peptides were obtained. The crude peptides were purified *via* semi preparative HPLC and characterized using NMR and HRMS.

1.3.3 General procedure for the preparation of *N*-oxide tetrapeptides.

Crude peptide (0.1 mmol) and K_2CO_3 (2.5 equiv.) were dissolved in dry DCM (10 ml). m-CPBA (1.6 equiv.) was added to a cooled solution mixture (-72 °C). The reaction was stirred for four hours under the same conditions and warmed up to room temperature over two hours. Finally, the solvent was evaporated and the product was purified by preparative HPLC and characterized with NMR and HRMS.

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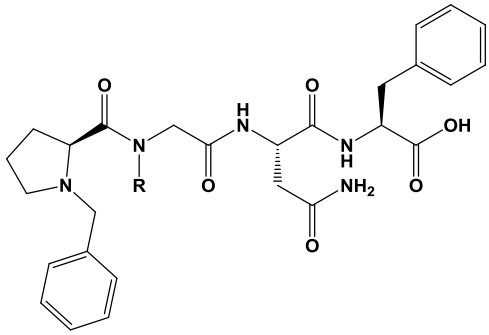
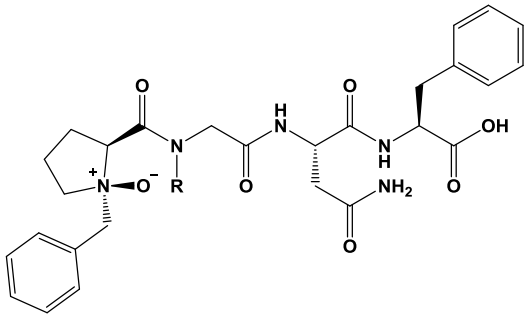
CHAPTER 3

2 Summary

Two groups of peptide were investigated in this study. The first group is designed to yield optimal turn characteristics. In contrast, the second group possess sterically hindered residues that were selected to prevent the formation of turns in their peptide backbones.

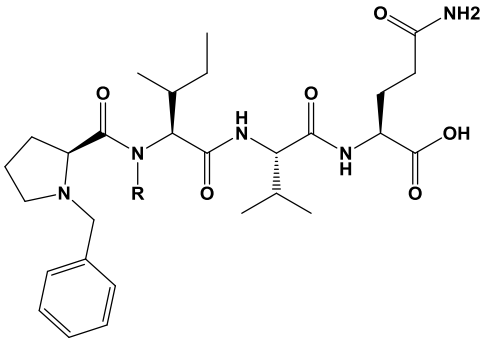
The members of first group were **1a,** (**BzPGNF**) and **2a,** (**BzPMeGNF**) and their corresponding *N*-oxide peptides **1b,** [**Bz**(**NO**)**PGNF**] and **2b,** [**Bz**(**NO**)**PMeGNF**]. Phenylalanine, asparagine and glycine were chosen for this backbone from the *C*-terminal to *N*-terminal respectively, and finally *N*-benzylproline was coupled to each.

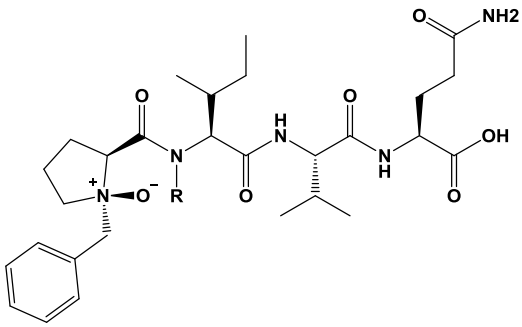




R: H **1a,** (**BzPGNF**) **1b,** [**Bz**(**NO**)**PGNF**]

R: Me **2a,** (**BzPMeGNF**) **2b,** [**Bz**(**NO**)**PMeGNF**]





R: H **3a**, (**BzPIVQ**) **3b,** [**Bz**(**NO**)**PIVQ**]

R: Me **4a,** (**BzPMeIVQ**) **4b,** [**Bz**(**NO**)**PMeIVQ**]

Figure 3.1. *N*-oxide-proline tetrapeptides.

As expected the amide groups of **1a** and **1b** possess *trans*-character. **1b** did not exhibit extraordinary NOE correlations, which suggests that this peptide is too flexible or perhaps assumes an extended conformation. The reaction between peptide **1a** and *m*-CPBA at -72°C led to the oxidation of the tertiary amine of *N*-benzylproline introducing a *N*-oxide moiety in the peptide sequence, to form *N*-oxide peptide **1b**. The ^{1}H NMR spectrum of this peptide reveals the hydrogen bonding interaction between the amide proton of glycine and oxygen of *N*-oxide, suggesting the presence of a quasi γ-turn in **1b**. A thermal coefficient NMR study was performed and the data confirmed this observation. Interestingly, **1b** exists as two conformers, one exhibiting a turn and another without. The population of the first conformer increases with elevated temperature suggesting that the turned structure is the kinetic product, while the proposed extended structure is the thermodynamic product.

Further investigations were undertaken to determine if this hydrogen bond could create larger turns or not. Therefore the first amide proton on glycine (i + 1) was blocked by *N*-methylation and thus peptide **2a** was obtained. In **2a**, *cis*- and *trans*-isomers with an approximate ratio of 47:53 % respectively, were detected from the ^{1}H NMR spectra. In addition, it seems that none of those isomers exhibited any backbone turns. The corresponding *N*-oxide peptide, **2b**, was successfully synthesized and elucidated. Peptide **2b** presented two isomers according to the ^{1}H NMR spectra with a *cis*- and *trans*-isomer ratio of 29:71 % respectively. The downfield back bone amide proton of asparagine (i + 2) in the *trans*-isomer (major conformation) suggests a quasi β-turn due this hydrogen bonding interaction. Additional thermal coefficient NMR experiments probing the nature of this hydrogen bond were performed.

The second class of peptides had the sequence glutamine (i + 3), valine (i + 2), isoleucine (i + 1) and *N*-benzylproline from *C*-terminal to *N*-terminal, respectively. Peptides **3a** and **3b** presented only *trans*-isomers and a quasi γ-turn was detected in **3b** (hydrogen bond interaction between the first amide proton next to proline and *N*-oxide). Interestingly, it seems that the presence of a bulkier amino acid in the (i + 1) position facilitates more stable hydrogen bonding interactions. Thermal coefficient NMR results supported this observation.

The final part of this study involved peptides **4a** and **4b** where they possess even more sterically hindered groups (*N*-methylated isoleucine was used as a (i + 1) residue). In the case of **4a**, we observed multiple isomers and the major and minor conformers were assigned as *cis*- and *trans*-isomers (40:60 %), respectively. In addition, a ROESY NMR experiment was used to elucidate each isomer and *N*-methyl proton integration was used to determine the ratio. Peptide **4a** did not

demonstrate any extraordinary NOE correlations, which suggests this sequence is either too flexible, or mostly in an extended form (β-sheet) in both isomers. After inserting the *N*-oxide to the backbone yielding **4b** as a product, a NMR investigation was performed to obtain a better understanding of the 3D structure of this peptide. Two isomers were observed resulting from a *cis*- and *trans*-isomerization process (77:23 %). The proton chemical shifts of **4b** (major/*cis*) reveal that the amide proton of valine (i + 2) is involved in a hydrogen bonding interaction with the *N*-oxide group. This contact is possible through a quasi β-turn, which is a novel achievement, especially in the presence of sterically hindered residues. In addition, it seems that the hydrogen bond occurred only in the *cis*-isomer perhaps explaining why it is observed as the major isomer, since stabilization of the secondary structure is increased through hydrogen bonding. This hydrogen bond interaction was confirmed with a thermal coefficient NMR study of this peptide.

Application of this novel structure characteristic in future studies should include testing of the bioactivity of such peptides as this turn decreases the peptide flexibility and the *N*-methylation should in principle increase the bioavailability. All of the details are summarized in the table following.

				Isomers				Thermal coefficient study with NMR					
Entry	**Sequence**	***N*-Me**	**NO$^-$**	***cis (%)***	***trans (%)***	**Turn (γ or β)**	**Presence of NOE correlations**	**(i + 1)**		**(i + 2)**		**(i + 3)**	
								cis	***trans***	***cis***	***trans***	***cis***	***trans***
1a	**BzPGNF**	-	-	-	100	-	-	-	2.35	-	5.80	-	1.73
1b	**Bz(NO)PGNF**	-	✓	-	100[b]	γ	-	-	-2.64[M]	-	4.56[M]	-	
								-	4.69[m]	-	4.71[m]	-	2.43[m]
2a	**BzPMeGNF**	✓	-	47	53	-	-	-	-	4.68	3.88	4.72	5.2
2b	**Bz(NO)PMeGNF**	✓	✓	29	71	β	✓	-	-	-[a]	-1.49[c]	-[a]	7.29
3a	**BzPIVQ**	-	-	-	100	-	-	-	5.52	-	5.63	-	2.21
3b	**Bz(NO)PIVQ**	-	✓	-	100	γ	-	-	-0.66	-	5.98	-	5.04
4a	**BzPMeIVQ**	✓	-	40	60	-	-	-	-	6.54	3.36	5.74	3.39
4b	**Bz(NO)PMeIVQ**	✓	✓	77	23	β	✓	-	-	0	-[a]	6.11	-[a]

[M] The major product at room temperature with *trans* amide bond for (i + 1).

[m] The minor product at room temperature with *trans* amide bond for (i + 1).

a This isomer was not elucidated due two low signal and overlapping with signal of major isomer.

b Two conformers with *trans*-isomer were assigned from ^{1}H NMR spectra.

c The minus value for -Δδ/ΔT represents the existence of acid-base equilibrium in this hydrogen bond.