4
Structure and Activity of *N*-Methylated Peptides

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4.1
Introduction

Peptides typically display high potency and target selectivity, making them valuable leads in the development of new therapeutics. Indeed, many peptides have made the transition to clinical use, including cyclosporine (cyclosporin A) [1, 2], gonadotropin-releasing hormone (also known as luteinizing-hormone releasing hormone) agonists and antagonists [3, 4], somatostatin analogs [3, 4], exenatide [5], ziconotide [6], and glatiramer acetate [7], to name just a few. Nonetheless, converting lead peptides to drugs represents a considerable challenge. Many peptides lack oral bioavailability as a consequence of their susceptibility to proteolysis in the gut, inefficient transport across the intestinal wall, proteolytic degradation in the bloodstream, and rapid clearance by the kidney.

Experience with the 35-residue polypeptide ShK toxin highlights the problem of renal clearance. This peptide, and analogs thereof [8], are potent immunosuppressants [9] that are of interest as therapeutic leads for the treatment of multiple sclerosis and other autoimmune diseases. One analog of ShK composed entirely of D-amino acids possessed a structure essentially identical to that of ShK [10], but was resistant to proteolysis [11]. This analog blocked the target potassium channel with nanomolar affinity and inhibited human T cell proliferation. Its immunogenicity was not tested, but it is reasonable to assume that if it could not be processed it is unlikely to be displayed by antigen-presenting cells. Despite these favorable attributes, the circulating half-life of D-allo-ShK was only slightly longer than that of ShK, implying that renal clearance was the major determinant of its plasma level. One potential strategy to circumvent this problem would be to encapsulate the peptide in a slow-release formulation that provides both predictable rates of release into the bloodstream [12, 13], and protection from peptidases and proteases. Another approach to prolong plasma half-life would be to couple the peptide to poly(ethylene glycol) (PEG) [14–16] or other partners [17, 18].

The first peptide therapeutic designed to target voltage-gated calcium channels (or indeed any ion channel), Prialt® (ziconotide) [19], illustrates several of the

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challenges facing peptide therapeutics. This 25-residue peptide, approved for severe chronic pain [20, 21], is a synthetic version of ω-conotoxin MVIIA. Prialt is delivered intrathecally via continuous delivery from a surgically implanted pump or from an external microinfusion device and catheter [6, 22]. However, it has a half-life in cerebrospinal fluid (CSF) of only 5 h and must be administered continually since the CSF replenishes at over triple its total volume each day. Continuous dosing requires implantation of a delivery system, which must be titrated by a physician in a hospital setting to obtain the correct dose and drug delivery rate. In addition, excess ziconotide in the bloodstream could reduce blood pressure through inhibition of calcium channels in sympathetic neurons [23]. Extending the half-life of ziconotide could allow administration by single injection, thereby eliminating the need for surgery to implant a pump for continuous infusion and facilitating dose titration, thus making the drug available to more patients. It is unlikely that glycosylation or attachment of PEG groups would extend the half-life of ziconotide in the CSF given the relatively rapid turnover of fluid from the CSF. A potential improvement would be to restrict the peptide to the spinal cord and exclude it from the brain, thereby possibly eliminating central nervous system (CNS) side-effects [19].

Several strategies have been developed to improve the efficacy of therapeutic peptides, one of which is methylation of backbone amides (*N*-methylation) (Figure 4.1). Introduction of a backbone *N*-Me group has been shown to substantially improve a number of pharmacokinetically useful parameters, including membrane

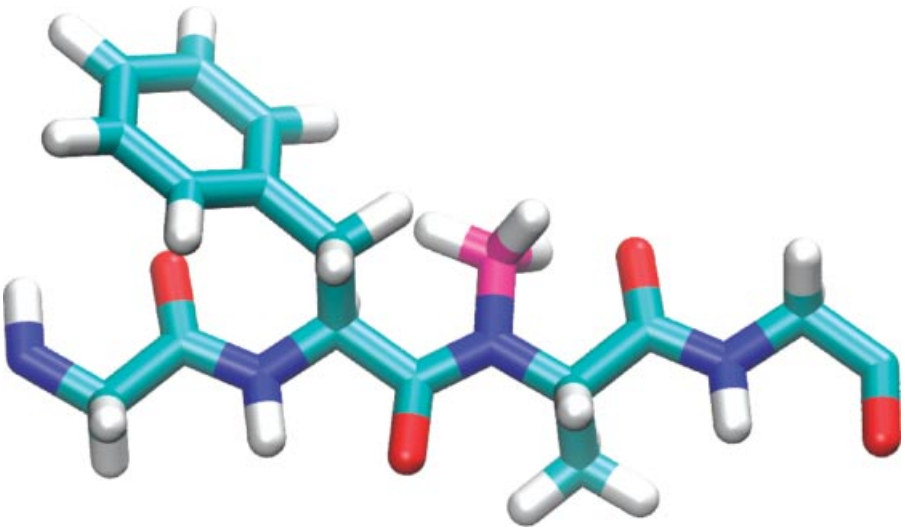

Figure 4.1 Schematic of a tetrapeptide unit consisting of Gly–Phe–(*N*-Me)Ala–Gly, with all peptide bonds in the *trans* configuration. Standard geometries were used in constructing this model, which has not been energy minimized. The *N*-Me group is highlighted in magenta.

permeability and proteolytic stability. Moreover, *N*-methylation results in a loss of hydrogen bonding potential at the affected site, reducing the role of main-chain hydrogen bonds at a binding interface and potentially altering binding properties [24]. Structurally, this modification largely restricts the affected residue and the amino acid preceding it to an extended conformation, as discussed in detail below. Modification of several biologically active peptides by the inclusion of *N*-Me amino acids into a sequence has been shown to enhance potency [25, 26], change receptor subtype selectivity [27, 28], and protect the peptide from proteolytic degradation [29]. In the pentapeptide ipamorelin, a highly potent and selective growth hormone-releasing peptide, several truncated and *N*-methylated analogs exhibited 10–20% oral bioavailability in animals [30]. This approach therefore offers the potential to overcome several potential limitations of peptides as therapeutics. *N*-Me substitutions have also proven useful in modulating the potency or selectivity of peptide ligands in the course of structure–function analyses.

4.2 Conformational Effects of *N*-Methylation

Manavalan and Momany [31] undertook empirical conformational energy calculations for *N*-Ac-*N*-Me-*N*′-Me-L-Ala-amide in both its *cis* and *trans* configurations. Introduction of the *N*-Me group in the *trans* configuration shifted the lowest energy position from $\varphi\ -80^\circ/\psi\ 80^\circ$ to $\varphi\ -120^\circ/\psi\ 70^\circ$. The right-handed helical region $\varphi\ -50^\circ/\psi\ -50^\circ$ was energetically forbidden, being 20 kcal/mol higher than the lowest energy state, and the second lowest energy state lay in the α_L region ($\varphi\ -50^\circ/\psi\ -50^\circ$), which is about 1 kcal/mol higher than the lowest energy position. The φ/ψ values obtained from the crystal structures of *N*-Me derivatives all fell within the 10 kcal/mol contours of the Ramachandran plots. In the *cis* configuration there were only two local minima, with the lowest energy state occurring near $\varphi\ -140^\circ/\psi\ 70^\circ$. Comparison of energy values between the *cis* and *trans* models at their minimum-energy positions showed that the peptide with a *cis* conformation was less stable than the *trans* by 4.5 kcal/mol. This energy difference was consistent with the experimentally observed preference for a *trans* configuration in poly(*N*-Me)-Ala [32].

In *N*-Ac-*N*-Me-*N*′,*N*′-diMe-L-Ala-amide, which serves as a model of *N*-methylation at both the i and $i + 1$ amide nitrogens, the low-energy regions were similar to those in *N*-Ac-*N*-Me-*N*′-Me-L-Ala-amide except that the areas were reduced within the 1 and 3 kcal/mol contours. The α_L state was elevated by 3 kcal/mol relative to the lowest energy position, making the region around $\varphi\ -140^\circ/\psi\ 80^\circ$ the most probable conformation. Similar conclusions were reached when the Ala side-chain was replaced with that of Phe. Energy calculations also showed that deviations from planarity for the peptide bond were more likely for *N*-methylated peptides than their unmethylated counterparts – a finding supported by crystal structures of cyclic peptides containing *N*-Me groups [33–35], which show deviations in ω ranging from 5 to 19°.

In summary, *N*-methylation reduces the energy difference between the *cis* and *trans* isomers, thereby increasing the probability that a *cis* peptide bond will be found at the site of *N*-methylation [36–38], and favors an extended backbone conformation. In multiply *N*-methylated peptides, steric hindrance and the reduction in hydrogen bond donors make it difficult to predict the conformation.

More recently, Tran *et al.* [39] described simulations using a newer force field of the conformational effects of *N*-methylation and other peptide modifications. Their predictions for the effects of methylating both peptide bonds in Ac-Ala-NHMe (Figure 4.2) are very similar to those of Manavalan and Momany [31] except for the steeper energy minima in the previous maps. Side-chain atoms past the C^{β} position do not restrict the backbone conformation (although in proteins β-chain formation favors residues such as Leu, so the φ/ψ maps show Leu to be even more extended than Ala), so the calculations for Ala are likely to be representative of *N*-Me-Val and *N*-Me-Leu in peptides [40].

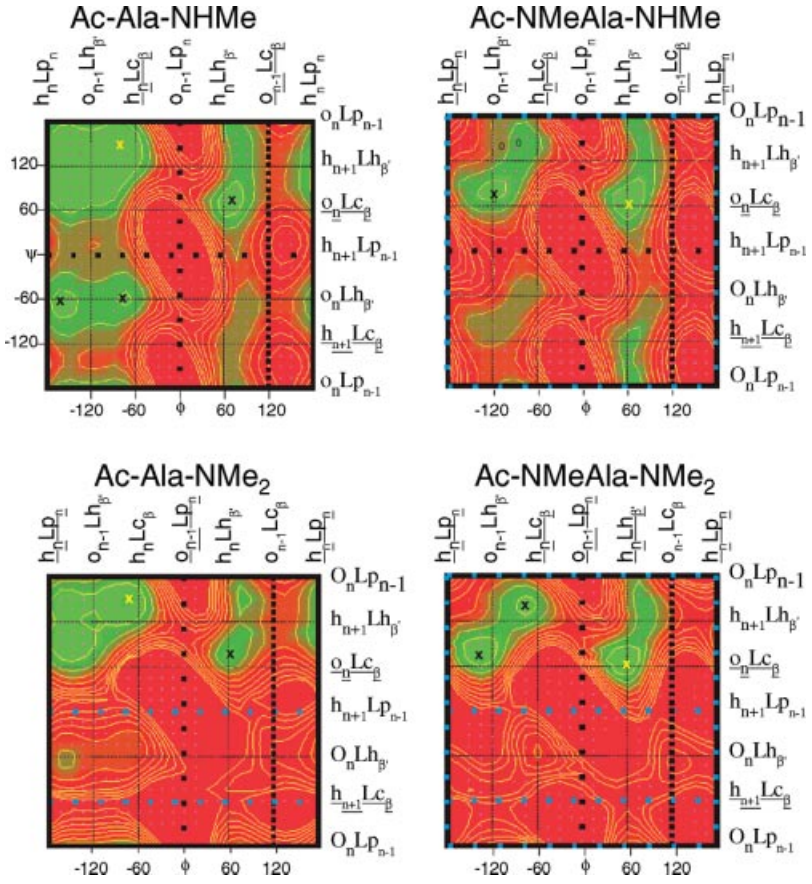

Figure 4.2 Conformational energy maps for Ala peptide models modified by increasing degrees of *N*-methylation. (Adapted from Figure 2 of [39].)

The 11-residue cyclic peptide cyclosporin A is an example of a multiply *N*-methylated peptide, with seven *N*-methylated residues, including one *N*-Me-Val and four *N*-Me-Leu residues [1]. The φ/ψ angles for the four *N*-Me-Leu at positions 4, 6, 9, and 10 in the recently determined structure of cyclosporin A in complex with human cyclophilin G (Protein Data Bank (PDB ID: 2WFJ) [41, 42] are $-114°/94°$, $-111°/177°$, $-129°/65°$, and $-111°/167°$, respectively. Thus, the φ values were quite tightly clustered and close to the values predicted from energy calculations, whereas the ψ values were more dispersed, but nonetheless all were in the predicted quadrant of a Ramachandran plot. The corresponding values for cyclosporin A in complex with human cyclophilin D (PDB ID: 2Z6W) are $-109°/93°$, $-120°/170°$, $-130°/69°$, and $-106°/169°$, respectively.

4.3 Effects of *N*-Methylation on Bioactive Peptides

4.3.1 Thyrotropin-Releasing Hormone

Manavalan and Momany [31] also described empirical conformational energy calculations for the tripeptide thyrotropin-releasing hormone (pGlu–His–Pro-NH_2, where pGlu indicates pyroglutamate) and its (*N*-Me)His2 analog. The *N*-methylated analog is equipotent with the native peptide in both *in vitro* and *in vivo* assays [43]. Two distinct low-energy conformers were predicted for the *N*-methylated peptide, separated by an energy difference of only 1.1 kcal/mol. The first conformation had negative φ_2 and positive ψ_2 values, and the second had positive φ_2 and ψ_2 values. The conformational space available to this analog was less than that available to the unmodified peptide [44]. Nuclear magnetic resonance (NMR) analyses of the (*N*-Me)His2 analog [43] in both aqueous and nonaqueous solvents [45] yielded φ_2 $-150°$ and φ_2 155°, which are in close agreement with the values calculated for the lowest energy conformation.

4.3.2 Cyclic Peptides

Kessler *et al.* [25] investigated the influence of *N*-methylation of the selective $\alpha_V\beta_3$ antagonist cyclo(RGDfV) (where lower case denotes a D-amino acid) on biological activity. Cyclo(RGDf-(*N*-Me)V) was found to be more active than the unmethylated peptide, and one of the most active and selective compounds in inhibiting vitronectin binding to $\alpha_V\beta_3$. Its structure in aqueous solution was determined from NMR data and molecular dynamics calculations [25]. The *N*-methylated peptide adopts a conformation characterized by a fast equilibrium between two inverse γ turns at Arg1 and Asp3 and a γ turn at Gly2. It was proposed that the *N*-Me group imposed steric repulsion via the peptide bonds Asp–D-Phe and Val–Arg, and blocked hydrogen bond formation between Arg1 HN and Asp3 CO, leading to a less-kinked orientation of the RGD pharmacophore.

Subsequently, an NMR study of the effect of *N*-methylation on a series of cyclic pentapeptides, cyclo(-D-Ala–L-Ala$_4$-) [38], showed that only seven out of the 30 compounds adopted a single conformation (on the NMR time scale of chemical shift separation), whereas the others displayed two or more conformations in slow exchange. Among those analogs that adopted a single conformation, the incorporation of an *N*-Me moiety in place of the NH group introduced relatively minor conformational changes where no steric clashes would arise. In contrast, where introduction of the *N*-Me group was not sterically allowed, the conformation changed by introduction of a *cis* peptide bond between Ala4 and Ala5. Notably, of the seven well-defined structures, six had the D-residue *N*-methylated, implying that this combination represents a potentially valuable means of specifying a preferred conformation. It was also observed that further *N*-methylation of the conformationally homogeneous peptides did not guarantee the existence of a preferred conformer on the NMR timescale, suggesting that the most promising templates for future rational design were those with either mono- or di-*N*-methylation.

4.3.3
Somatostatin Analogs

The tetradecapeptide somatostatin exerts potent inhibitory effects on secretory processes in tissues such as pituitary, pancreas, or gastrointestinal tract, as well as acting as a neuromodulator in the CNS. These biological effects are elicited by inhibition of a series of G-protein-coupled receptors, of which five different subtypes have been characterized; these subtypes have similar affinities for the ligand but different distribution in various tissues [46]. *N*-Methylation at Lys9 of a somatostatin analog based on octreotide (sandostatin; D-Phe5-cyclo[Cys6-Phe7-D-Trp8-Lys9-Thr10-Cys11]-Thr12) enhanced potency by around 4-fold and modified receptor subtype selectivity [27]; this was somewhat surprising given that Lys9 has been considered to constitute the active center of somatostatin, but may indicate that the introduced *N*-Me group makes favorable interactions with the receptor and/or stabilizes the peptide in its bound conformation.

Previously, Veber *et al.* [26] demonstrated that the mono-*N*-methylated cyclic somatostatin analog cyclo[(*N*-Me)Ala6-Tyr7-D-Trp8-Lys9-Val10-Phe11] had 50- to 100-fold greater potency than somatostatin in the inhibition of insulin, glucagon, and growth hormone release. This hexapeptide showed good metabolic stability, but only limited oral bioavailability.

Kessler *et al.* recently undertook a complete *N*-Me scan of the cyclopeptidic somatostatin analog cyclo[Pro6-Phe7-D-Trp8-Lys9-Thr10-Phe11], known as the Veber–Hirschmann peptide [47, 48]. They synthesized and characterized 30 *N*-methylated analogs. Screening of these analogs against the human receptor subtypes hsst$_{1-5}$ showed that seven had affinities similar to that of the parent peptide (i.e., nanomolar affinity for receptor subtypes hsst$_2$ and hsst$_5$). In all seven of these active analogs, the βII′ and the βVI turns were conserved (as determined by NMR spectroscopy and molecular dynamics calculations), confirming the importance of these two turns in maintaining the peptide in its bioactive conformation. There was

also no significant conformational perturbation associated with *N*-methylation, even at multiple sites.

In addition, no significant degradation was observed for any of the *N*-methylated peptides after 7 h incubation in rat serum. An analog triply *N*-methylated at D-Trp8, Lys9, and Phe11 retained receptor-binding activity, was not degraded by digestive enzymes isolated from the brush border, and showed the highest intestinal permeability in an *in vitro* model. Importantly, following administration of this analog by oral gavage at a dose one order of magnitude higher than the intravenous dose (i.e., 10 versus 1 mg/kg), oral bioavailability was found to be 10%, in contrast to the complete lack of oral bioavailability for the parent peptide [47].

4.3.4 Antimalarial Peptide

The 20-residue peptide, R1, is a potent inhibitor of malaria parasite invasion of red blood cells [49]. R1 is an important lead compound for drug development, because its ability to block parasite growth indicates that it targets a site critical for apical membrane antigen-1 (AMA1) function. However, this peptide inhibitor is only effective against a limited subset of parasite isolates and does not exhibit broad strain specificity. To address this problem, Foley *et al.* [50] sought to improve the proteolytic stability and AMA1 binding properties of R1 by systematic methylation of backbone amides. The inclusion of a single *N*-Me group increased AMA1 affinity (Table 4.1), bioactivity, and proteolytic stability without introducing global structural alterations (as assessed by NMR). In addition, *N*-methylation of multiple R1 residues further improved these properties, as summarized in Table 4.1.

In this study, the deviations of backbone NH and $C^{\alpha}H$ chemical shifts from random coil values were used as a proxy for conformational changes associated with *N*-methylation. Plots of the differences between these shifts for each peptide and corresponding values for native R1 showed that the differences caused by *N*-methylation were predominantly local, mostly within two to three residues either

Table 4.1 Equilibrium constants determined by surface plasmon resonance for the interaction of *N*-methylated R1 derivatives with different strains of *P. falciparum* AMA1 [50].

AMA1 variant peptide	3D7 K_D (nM)[a]	3D7 K_D (nM)[b]	W2mef K_D (μM)[b]	HB3 K_D (μM)[b]
R1	77	80	17	69
[(*N*-Me)Leu8]-R1	23	ND[c]	15	55
[(*N*-Me)Leu8/(*N*-Me)Ser14]-R1	11	ND[c]	6	24
[(*N*-Me)Val1/(*N*-Me)Leu8/(*N*-Me)Ser14]-R1	13	ND[c]	6	25

a) K_D estimated using a kinetic analysis of Biacore data, as described by Harris *et al.* [50]. All K_D values in this table have been rounded to the nearest integer; exact values and errors are given in Harris *et al.* [50].

b) K_D estimated using a steady-state analysis of Biacore data, as described by Harris *et al.* [50].

c) ND = not determined.

side of the *N*-Me substitution, implying that *N*-methylation did not cause any long-range structural changes in R1. Calculated structures were consistent with the previously described solution structure of R1, which consists of two structured regions, both involving turns; the first of these, encompassing residues 5–10, is hydrophobic and the second, involving residues 13–17, is more polar [49]. Even in solution these turns are unlikely to represent the only conformations sampled by the peptide, because linear peptides lacking any covalent cross-links, as in the case of R1, are known to sample a range of rapidly interconverting conformations in aqueous solution.

Harris *et al.* [50] also addressed whether the *N*-Me groups nucleated local structure (as opposed to long-range structure, which is effectively ruled out by the lack of extensive chemical shift changes). A confounding factor here is that *N*-Me groups give sharp, strong NMR resonances, which are likely to detect interproton nuclear Overhauser effects (NOEs) over longer distances than the parent backbone amide proton. Structures were therefore calculated for various *N*-Me analogs in the presence and absence of NOEs to the *N*-Me groups. In essence, the structures of *N*-Me analogs calculated without *N*-Me NOEs were similar to the structure of R1, but inclusion of *N*-Me NOEs and two long-range NOEs in the structure calculations for the *N*-Me-Leu-8 analog caused an apparent stabilization of structure in the vicinity of the *N*-Me group, resulting in an apparently more compact global structure. These local effects have to be considered in NMR analyses of linear peptides such as R1, where there is a dearth of long-range NOEs and an absence of covalent constraints.

The enhanced affinity for AMA1 and broader strain specificity exhibited by a number of the *N*-Me R1 analogs implies that neither intra- nor intermolecular hydrogen bonding interactions play a critical role in R1 binding to AMA1. As the binding site for R1 on AMA1 is likely to be a hydrophobic groove (Richard *et al.*, unpublished results), the reduction in peptide polarity associated with *N*-methylation may contribute to the higher affinity. The combination of higher affinity, broader strain specificity, and resistance to proteolysis in plasma [50] makes the *N*-methylated analogs of R1 an attractive starting point for further development.

4.4 Concluding Remarks

There are many ways in which *N*-methylation can affect a peptide's conformation, affinity, selectivity, stability and bioavailability. *N*-Methylation could interfere with amide group hydrogen bond formation, or the free energy of binding could be affected as a result of the introduction of a hydrophobic methyl group or the imposition of steric hindrance. Perturbation of the local conformation, although unlikely to be major, may be sufficient to modulate affinity. The overall effect of *N*-methylation on affinity will reflect a balance across all of these factors. For example, in the *N*-methylated R1 analogs, altered local conformation, loss of hydrogen bonding, and steric repulsion will contribute to the loss of binding affinity observed for some analogs. In contrast, for those analogs with higher affinity, *N*-methylation

presumably favored the bound conformation of the peptide, while hydrophobic interactions between introduced *N*-Me groups and the nonpolar binding groove of AMA1 may also contribute.

N-Methylated analogs with the desired affinity and target specificity identified by *N*-Me scanning are likely to have other beneficial properties: resistance to proteolysis in the gut and bloodstream, reduced polarity, and enhanced bioavailability and pharmacokinetics are all potential attributes of *N*-methylated peptides that may be expected to make them attractive analogs for further development of peptides as therapeutics. The immunosuppressant drug cyclosporine (cyclosporin A), which is widely used in transplantation to reduce the risk of organ rejection, provides an excellent example of a naturally occurring peptide with a high degree of *N*-methylation and good bioavailability.

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