UNIVERSITY OF CALIFORNIA
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SELECTIVE, ON-RESIN N-METHYLATION OF CYCLIC PEPTIDES
AND IMPLICATIONS FOR THE DISCOVERY OF MEMBRANE
PERMEABLE SCAFFOLDS

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY AND BIOCHEMISTRY

by

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June 2012

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Abstract

Tina White

Selective, on-resin N-methylation of cyclic peptides and implications for the discovery of membrane permeable scaffolds

Many biologically active cyclic peptides, especially those of natural origin, are known to penetrate cells by passive diffusion despite lying well outside Lipinski’s “rules of 5” for predicting bioavailability. The backbone amides of these natural products are often N-methylated, a modification that serves to enhance lipophilicity and proteolytic stability. The impact of N-methylation on passive membrane diffusion in cyclic peptides, however, has not been investigated in detail. Here we present a new method for the selective, on-resin N-methylation of cyclic peptides to generate compounds with drug-like membrane permeability. The selectivity and degree of N-methylation of the cyclic peptide depends on the stereochemistry of the backbone, suggesting that conformation dictates the regiochemistry of the N-methylation reaction. For two of the diastereomers investigated, unique N-methyl products were observed with each diastereomer yielding a different N-methyl variant. 2D 1H NMR and H/D exchange studies provide insight into the conformational basis of the regioselectivity of the reaction and provide a rationale for the observation that the partially methylated derivatives are not only significantly more membrane permeable than their nonmethylated precursors, but are also more permeable than the corresponding permethylated species.
Acknowledgments

Tina White synthesized the diastereomer library, developed on-resin N-methylation chemistry, performed PAMPA studies, and synthesized peptide library and the leucine-to-serine analogs. Chad Renzelman performed NMR experiments and analyzed data to determine patterns of N-methylation. Arthur Rand synthesized and performed H-D exchange studies on compound 7. Taha Rezai contributed to the development of the on-resin N-methylation chemistry. Cayla McEwen coordinated experiments and contributed to manuscript preparation. Vladimir Gelev assisted in analysis of 2D NMR data. Rushia Turner contributed to synthesis method development and analytical procedures for PAMPA and compound characterization. Roger Linington provided guidance in developing NMR methods to determine pattern of N-methylation. Siegfried Lung performed computational studies, generated virtual libraries and predicted permeabilities of virtual compounds. Amit Kalgutkar, Jonathan Bauman, Yizhong Zhang, Spiros Liras, David Price and Alan Mathiowetz designed pharmacokinetic studies and interpreted results of in vitro and in vivo absorption, distribution, metabolism and excretion and pharmacokinetic data. Matthew Jacobson developed computational methodology for predicting permeability, designed experiments and discussed results. R.S.L. conceived the on-resin N-methylation approach and designed experiments. Amit Kalgutkar, J.N.B.,
Jonathan Bauman, Yizhong Zhang, Spiros Liras, David Price Alan Mathiowetz, Matthew Jacobson and Scott Lokey discussed results and wrote the paper.
Chapter 1

1.1 N-methylated Cyclic Peptides as Pharmacologically Important Scaffolds

Cyclic peptides constitute a large class of natural products with a wide array of cellular targets and biological effects. Comprised of readily available amino acid starting materials, their structures are widely variable in chemical characteristics, conformation, and stereochemistry, because of their cyclic nature they are resistant to enzymatic degradation\(^1\). As such they are very desirable synthetic targets for pharmacological studies.

Due to their large size, cyclic peptides frequently exhibit low solubility and low cell permeability, defying Lipinski’s rules both in molecular weight and number of hydrogen bond donors and acceptors. Peptides are susceptible to degradation by peptidases in the digestive tract, and in general have poor intestinal permeation. In addition, the instability of peptides toward peptidases in blood circulation causes rapid elimination from the bloodstream. Cyclic peptides exhibit increased chemical stability and longer \textit{in vivo} half-life compared to their linear counterparts. However, low oral bioavailability decreases their usefulness as therapeutic agents in the clinical setting. Because of this, focus has
been directed towards modifying cyclic peptide structures to increase cell permeability and bioavailability. One modification frequently observed in natural products is N-methylation. N-methylation of the peptide backbone increases the general lipophilicity by reducing the number of hydrogen bond donors within the molecule. However, because of the conformational effects of stereochemistry, backbone rigidity afforded by N-methylation, and conformational restrictions due to cyclization, the

**Figure 1.** A) Non-methylated RGD cyclic peptide. B) Hydrogen bonding pattern of the native cyclic peptide with solvent-exposed amide protons highlighted. C) Most selective N-methylated peptide with solvent-exposed amides methylated.
hydrogen bond donors and acceptors that are remaining are often involved in intramolecular hydrogen bonds. This effectively reduces the number of donors and acceptors, further and increasing cell permeability (Figure 1)[2].

N·methylation can increase potency of cyclic peptide ligands. Sansalvamide A, a cyclic pentadepsipeptide natural product, was found to be cytotoxic against colon and melanoma cancer cell lines (Figure 2a)[3]. However, the natural product shows little activity against pancreatic cancer cell lines. Via structure·activity relationship studies, analogues of Sansalvamide A were synthesized that showed higher potency against

![Figure 2. A) Sansalvamide A, cyclo(VLFL(O)L)](A) B) The most potent N·methyl peptide analogue of sansalvamide A
several tumor cell lines\cite{4}. Upon further investigation, a single analogue of Sansalvamide A (Figure 2b), consisting of all peptide bonds and one N-methylated amide bond, was shown to have a time- and concentration-dependent inhibition of DNA synthesis and cell proliferation in two human pancreatic cancer cell lines. It was determined that this N-methylated analogue induced apoptosis after inhibiting cell growth via G0/G1 arrest\cite{5}.

N-methylation has been shown to improve receptor selectivity. The drug Cilengitide, cyclo(RGDf(Me)V) (Figure 3a), is a cyclic RGD

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{A) Cilengitide, Cyclo(RGDf(Me)V) B) Most selective di-N-methylated analogue, Cyclo((Me)RG倪f(Me)V) C) Activity of Cilengitide and the di-N-methylated analogue}
\end{figure}

\begin{table}
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\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
 & \(\alpha v3\) & \(\alpha v5\) & \(\alpha 5β1\) & \(\alpha llβ3\) & \(\frac{\alpha 5β5/\alpha v3}{\alpha 5β1/\alpha v3}\) & \\
\hline
Cilengitide & 0.65\((+/-.07)\) & 11.7\((+/-.15)\) & 13.2\((+/-.6)\) & 815\((+/-.60)\) & 18 & 20 \\
Dimethyl & 5.9\((+/-.2.5)\) & >3000 & 270\((+/-.95)\) & >1000 & >500 & 46 \\
\hline
\end{tabular}
\end{table}
pentapeptide currently in phase III clinical trials for the treatment of brain tumors and in phase II for other cancer types\cite{6}. The antitumoral properties of this peptide are based on its antagonistic activity for pro-angiogenic integrins, such as αvβ3, αvβ5, and α5β1. As a combinatorial library, all possible di-N-methylated analogues of the peptide scaffold cyclo(RGDfV) were synthesized. This study yielded a single regioisomer of di-N-methylated cilengitide (Figure 3b) which retained αvβ3-binding activity in the nanomolar range but lost activity towards integrins αvβ5.
and α5β1. Highly active and selective peptides for αvβ3 can be used to study the role of this particular integrin in angiogenesis and cancer\textsuperscript{[7]}. In this case, adding a single N-methyl to the already singly N-methylated peptide scaffold generated a product that was orders of magnitude more selective for a single integrin target than the singly methylated scaffold. A similar study of MK-678, a cyclic hexapeptide derivative of the pharmacophore of somatostatin, also demonstrated how N-methylation can affect selectivity. Here, a designed approach was used. Rather than generating a combinatorial library of all possible N-methyl analogues, the solution structure of the peptide was used to determine which amide protons were solvent exposed and only those positions were selectively N-methylated. Of the 32 possible N-methyl analogues, only 7 were synthesized. Of these 7, one tetramethylated analogue (Figure 4) showed a large increase in selectivity for the somatostatin receptor sst2 over sst1, sst3, sst4, and sst5. This selectivity was higher than both the natural product somatostatin and the synthetic MK-678 derivative\textsuperscript{[8]}.

N-Methylation restricts the available φ/ψ space for the peptide backbone, yielding a more rigid backbone conformation. Using dipeptide models, including both \textit{cis} and \textit{trans} peptide bonds, Ramachandran plots of the φ and ψ angles of N-methylated peptides were examined. The energy for each model system was calculated at 10° intervals over the
complete $\phi/\psi$ space. The allowed dihedral angles for N-methylated peptides differed from those for normal amide bonds. Introduction of an N-methyl group shifts the lowest energy position from ($\phi \sim -80^\circ$, $\psi \sim 80^\circ$) to ($\phi \sim -130^\circ$, $\psi \sim 70^\circ$). The right-handed helical region is energetically forbidden in these model systems, with a $\sim 20$ kcal/mol difference in energy from the lowest energy state. Overall, the effect observed is that the general shape of the isoenergetic contours are not altered significantly by N-methylation, but simply inhabit a smaller region of $\phi/\psi$ space, yielding a restricted conformation that is more rigid than a non-methylated peptide backbone\cite{9}.

N-Methylation can be employed to improve oral bioavailability, affecting bioavailability markers such as solubility and chemical stability against temperature and pH changes. In a study by Gordon, et al, a linear pentapeptide homologue of the Alzheimer’s $\beta$-amyloid peptide core domain (Ac-K(Me)LV(Me)FF-NH$_2$) was methylated at alternating positions (Figure 5). This peptide adopts an extended, $\beta$-strand conformation, yielding a peptide with two different faces. One side of the peptide exhibits a normal peptide backbone capable of hydrogen bonding through the amide protons; the other side has limited hydrogen-bonding capabilities due to N-methylation of the amides. The structure of this peptide is remarkably stable to changes in solvent conditions, resists
denaturation by heating, changes in pH, and addition of denaturants such as urea and guanidine-HCl. It is highly water soluble at concentrations up to 0.30 mM, in contrast to the non-methylated counterpart, even though it contains more hydrophobic moieties. The peptide is also able to pass spontaneously through both synthetic phospholipid bilayer vesicles and cell membranes\[^{10}\]. In this instance, selective methylation of even a linear peptide sequence increased permeability, stability, and bioavailability.

Both degree of N-methylation and position of N-methylation on the backbone effect intestinal permeability. In a recent study, a near exhaustive N-methylation library of an all-alanine model peptide scaffold was synthesized in order to determine the effect of degree of methylation
and position on intestinal permeability. The library consisted of an all- alanine cyclic hexapeptide with a single D-alanine residue, selectively methylated for a variety of methylation patterns, yielding 54 scaffolds. Of these 54 N-methylated analogues, 10 were found to have transcellular permeability similar to testosterone, a common intestinal permeability marker, as determined using the Caco-2 model. The permeabilities of all synthesized di-N-methylated peptides are shown in figure 6. The permeabilities of these di-N-methylated peptides vary greatly, indicating the importance of positional scanning of regioisomers. None of the tested peptides was found to permeate the PAMPA_{lecithin} artificial membrane, which reduces the possibility that the N-methylation facilitated the peptide’s transcellular permeability as a result of enhanced lipophilicity.

![Figure 6](image_url)
In this study, there was no direct correlation between permeability and degree of methylation; however, permeability was highly dependent on the position of the N-methyl groups. Of the 10 analogues that were found to be permeable, 9 contained an N-methyl group adjacent to the D-alanine residue\textsuperscript{[11]}. 
1.2 N-methylated Cyclic Peptides

1.2.1 Tetrapeptides

Hirsutide, derived from the fungus *Hirsutella* sp. BCC 1528, is a cyclic peptide with four amino acids, two of which are methylated (Figure 7). The amino acids are all L-configuration stereochemistry and naturally occurring. It was found to display moderate cytotoxicity ($IC_{50} = 11 \mu g/mL$) against P388 murine leukemia cells[12].

![Figure 7. Hirsutide Cyclo-(Val-(Me)Phe-Phe-(Me)Phe)](image)

Tentoxin, derived from the fungus *Alternaria tenuis*, is a cyclic tetrapeptide with a single non-standard amino acid, N-methyl-dehydrophenylalanine (Figure 8). Half of the amides in this scaffold are N-methylated. Tentoxin is being researched as a potential selective herbicide. It has been found to induce chlorosis, a condition in which plants lack sufficient chlorophyll, in dicotyledon plants[13].

![Figure 8. Tentoxin Cyclo-((Me)-DehydroPhe-Gly-(Me)Ala-Leu)](image)

It has also been the focus of combinatorial libraries[14].
1.2.2 Pentapeptides

Cotteslosin A and B are cyclic pentapeptide natural products, derived from the marine isolates of the fungus *Aspergillus versicolor* (MST-MF495). They have been found to be weak cytotoxic agents against human cancer cell lines (EC$_{50}$ $\sim$ 75 μg/mL). Both contain all L-configuration amino acids, with one amino acid bearing an N-methyl group and containing a single proline. Cotteslosin B contains a non-standard amino acid, L-allo-isoleucine at postion 4 (Figure 9)[15].

Bingchamide A and B were derived from organic extracts of the mycelium from the soil bacterium *Streptomyces bingchenggensis*. The all-L amino acid scaffold contains a single N-methyl group. Bingchamide B contains a non-standard amino acid, 3-(furan-3-yl)-alanine. Both peptides showed a dose-dependent inhibition of growth of human colon carcinoma cell line HCT-116 (IC$_{50}$ $\sim$ 15μg/mL) (Figure 10)[16].
The lichen *Leptogium saturninum* was found to harbor the endolichenic fungus *Xylaria* sp. (75-1-3-1). Crude extracts from this species yielded a novel cyclic pentapeptide containing a single N-methylated backbone amide and a single D-amino acid. This peptide exhibited synergistic antifungal activity against *Candida albicans* SC5314 at 6.25 μg/mL with 0.004 μg/mL ketoconazole (Figure 11)[17].
Cilengitide is a synthetic cyclic peptide, developed from a combinatorial library of “RGD” pentapeptides containing a single N-methyl group and the key arginine-glycine-aspartic acid (RGD) sequence. Cilengitide is in phase III clinical trials for the treatment of glioblastomas and in phase II for other tumor types. It has been found to be angiogenic, targeting integrins αvβ3, αvβ5, and α5β1. Many integrins recognize the RGD motif; however, they are able to discriminate among natural ligands. It is postulated that the conformation, afforded here by N-methylation, cyclization, and incorporation of a D-residue, is responsible for the selectivity of Cilengitide toward specific integrins (Figure 12)[6][18].

Sansalvamide A is a pentadepsipeptide derived from the fungus Fusarium sp. CNL 292, sequence Cyclo(Phe·Leu·(O)Leu·Val·Leu)[3]. A combinatorial library generated by eliminating the depsipeptide bond and replacing it with an amide bond, then selectively methylating the backbone amides yielded a derivative of Sansalvamide A that was more potent than the natural product. The N-methylated peptide derivative
Sansalvamide A has an IC\textsubscript{50} of 2.6 μg/mL, whereas sansalvamide A has an IC\textsubscript{50} of 3.5μg/mL against HCT 116 human colon cancer cell lines (Figure 13)\cite{19}.

Derived from marine algae Galaxaura filamentosa, Galaxamide A is a di-N-methylated cyclic all-leucine peptide. It has been found to be active against human renal cell carcinoma GRC-1, and human hepatocellular carcinoma HepG2 cell lines with IC\textsubscript{50} values of 4.26 and 4.63 μg/mL, respectively (Figure 14)\cite{20}.
Persipeptide A and B were isolated from *Streptomyces* sp. UTMC 1154 (Figure 15).

Containing all naturally occurring L-amino acids, they differ only in the transposition of N-methyl-valine and N-methyl phenylalanine. These scaffolds have shown no activity against tested algae, fungus, or bacteria[21].

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<tr>
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<td>CH(CH₃)₂</td>
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<tr>
<td>B</td>
<td>CH(CH₃)₂</td>
<td>CH₂(C₆H₅)</td>
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**Figure 15.** Persipeptide
A: Cyclo((Me)Phe\-Val\-Phe\-(Me)Val\-Val)
B: Cyclo((Me)Val\-Val\-Phe\-(Me)Phe\-Val)

1.2.3 Hexapeptides

NW·G01 and NW·G03 are cyclic hexapeptides isolated from *Streptomyces alboflavus 313* (Figure 16). Their structures are very similar, including the non-standard amino acid piperazic acid in both stereochemical configurations, a single D-amino acid, and a chlorinated pyrroloindoline derivative. It is worth noting that the inclusion of the non-standard, backbone cyclized amino acid side chains and the single N-methyl group leaves only a single free amide. Both scaffolds were active against MRSA, with MIC values of 7.81 μg/mL for NW·G01 and 6.13
Figure 16. NW-G01 and NW-G03
NW-G01: Cyclo(D-Val-(R)Pip-(S)Pip-(Me)Ala-(S)Pip-Chhi)
NW-G03: Cyclo(D-val-(R)6-ene-Pip-(S)Pip-(Me)Ala-(S)Pip-Chhi)
Where Pip is piperazic acid and Chhi is (3aR,8aR)-6-chloro-1,2,3,3a,8,8a-hexahydro-3a-hydroxypyrrolo[2,3-b]indole-2-carboxylic acid

µg/mL for NW-G03, and both scaffolds were found to be ineffective against gram-negative bacteria. NW-G03 was also tested against three tumor cell lines and found to inhibit growth in all three\textsuperscript{[22][23].}

Developed from a combinatorial library based on VLA-4 (α4β1 integrin) inhibitors, peptide ZD-7349 is a singly methylated cyclic hexapeptide (Figure 17). It was shown to inhibit MOLT-4 cell adhesion to fibronectin with an IC\textsubscript{50} of 260nM and inhibits VCAM-1 with an IC\textsubscript{50} of 330nM, though it showed
no appreciable activity against other integrins, making this scaffold highly selective[24].

Seglitide, also known as MK-678, is a cyclic hexapeptide derivative of the pharmacophore of somatostatin. It was shown to be a selective, though not highly potent somatostatin receptor antagonist[25]. Further studies found a tetramethylated derivative of seglitide that was more potent and selective[8].

The bacteria *Streptomyces silvensis* has yielded several cyclic peptide scaffolds that are potent oxytocin antagonists[26][27]. Based on these scaffolds, combinatorial libraries have been synthesized in order to

![Figure 18. MK-678, Seglitide Cyclo(Tyr-D-Trp-Lys-Val-Phe-(Me)Ala)](image)

Figure 18. MK-678, Seglitide Cyclo(Tyr-D-Trp-Lys-Val-Phe-(Me)Ala)

L-365209: Cyclo(Pro-D-Phe-Ile-(S)Dehydro-Pip-(R)-Dehydro-Pip-(Me)-D-Phe)
L-366811: Cyclo(Pro-D-Trp-Ile-(D)-Pipecolic acid-Piperazine-2-carboxylic acid-(Me)-D-Phe)

![Figure 19. L-365209 and L-355811](image)
increase selectivity and potency of the natural products. From these libraries, the scaffolds L·365209 and L·366811 have shown the most promising biological activity profiles. L·365209 is a potent oxytocin receptor antagonist, and could have potential utility as a selective agent for the prevention of premature birth\textsuperscript{[28]}. L·366811 exhibits significant bradykinin agonist activity, and stimulates phosphatidylinositol turnover in rat uterus slices \textit{in vitro} (EC\textsubscript{50} = 2 μM)\textsuperscript{[29]}.

Isolated from the soil actinomycine strain \textit{Amycolatopsis} sp. ML1·hF4, pargamicin A is a di·M·methylated cyclic hexapeptide containing four non-standard amino acids (Figure 20). It has highly potent bactericidal effects, with an MIC of 2 μM against \textit{Staphylococcus aureus} and 1μM against \textit{Enterococcus faecalis}. It has been shown to perturb cell membrane potential and function\textsuperscript{[30]}.

\textbf{Figure 20.} Pargamicin A Cyclo(Phe·(OH)Ile·Pip·(Me)3·Hydroxy·Val·4·Hydroxy·Pip·Sar)
Figure 21. NFI-028
Cyclo((2S, 4R)-2-amino-4-methyloctanoic acid-D-Piperidyl-Asp-(2S, 4R)-2-amino-4-methyloctanoic acid-(Me)Leu-Leu-(Me)Trp(N-Methoxy))

HUN-7239 is a methylated cyclic heptadepsipeptide with anti-inflammatory properties\cite{31}. Based on this scaffold, combinatorial libraries have been synthesized, improving selectivity and potency of the natural product. Derived from one of these libraries, NFI-028 is a Cyclic hexapeptide derivative of HUN 7239 with two N-methyl groups, and a potent inhibitor of VCAM-1 expression\cite{32}.

Scleramide is a cyclic hexapeptide isolated from sclerotia of \textit{Aspergillus sclerotiorum}. Discovered while searching for antiinsectans, this novel cyclic peptide did not show any activity (Figure 22)\cite{33}.

Figure 22. Scleramide
Cyclo(Phe-(Me)Phe-Gly-Gln-(Me)Phe-Gly)
The rubiyunnanin family of cyclic hexapeptides and the RA peptides I-XXIV, as well as bouvardin, are all structurally similar cyclic hexapeptides with a second ring structure formed between adjacent tyrosine residues. *Rubia akane* and *Rubia cordifolia* yielded the RA peptides, while *Rubia yunnanensis* is the source of the rubiyunnanins and bouvardin derived from *Bouvardia ternifolia*, all members of the rubiceae family of flowering plants. The rubiyunnanins (Figure 23) are cytotoxic against a panel of eleven cancer cell lines, with IC$_{50}$ values ranging from 0.001 to 56.24 μM, as well as inhibiting nitric oxide production and TNF-α-induced NF-κB activation$^{[34][35]}$. The RA peptides (Figure 24 and 25) have shown potent anti-tumoral properties, many with
nanomolar IC$_{50}$ values$^{[36][37][38][39][40][41][42][43][44]}$. The anti-tumoral properties have been shown to be highly conformation dependent after derivitization of the scaffold revealed that alteration of either the N-methylation pattern or the strained side-chain ring reduced activity. Bouvardin has been extensively studied and shown to be efficacious against colon adenocarcinoma, to inhibit protein synthesis through binding to the 80S ribosome, and to inhibit both amino acyl-\(t\)RNA binding and peptidyl-\(t\)RNA translocation$^{[45]}$.

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**Figure 24.** RA-IX, RA-XIV, and RA-VI
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</table>

**Figure 25.** Structures of related RA peptides, Bouvardin, and Rubiyunnanins
1.2.4 Heptapeptides

Di-N-methylated cyclic hexapeptides ilamycin A, B, and C were isolated from *Streptomyces islandicus*. These natural products have shown to be antibiotics against mycobacteria\[46\]. Rufomycin A and B were also isolated from a strain of *Streptomycetes*, and act as antibiotics against mycobacteria, including strains resistant to isonicotinic acid hydrazide, streptomycin, and kanamycin\[47\].

![Figure 26. Rufomycin A and B and Ilamycin A, B, and C](image_url)
Isolated from the broth of a marine fungus *Scytalidium* sp. CNC-310, scytilamides A and B are cyclic heptapeptides containing an unusual α-aminoisobutyric acid (Aib) residue (Figure 27), and differ only in the δ-methylated proline at position 1. Both scaffolds showed activity against the HCT-116 cell line with IC50 values of 2.7 and 11.0 μM, respectively[48].

![Scytilamide A and B](image_url)

**Figure 27.** Scytilamide A and B
A: Cyclo(Pro·(Me)Leu·Aib·Phe·(Me)Phe·Phe·Leu)
B: Cyclo(5·methylPro·(Me)Leu·Aib·Phe·(Me)Phe·Phe·Leu)
Isolated from marine bacteria *Streptomyces* sp. CNB-982 (A, B, and C) and *Salinospora arenicola* CNS-205 (D), the cyclomarins are cyclic heptapeptides comprised of a number of modified and non-standard amino acid residues (Figure 28). Cyclomarin D is known to display modest HCT-116 cytotoxicity with an IC\(_{50}\) of 2 \(\mu\)g/mL and no antifungal or antimicrobial activity\(^{[49]}\), while cyclomarin A acts as a potent anti-inflammatory agent\(^{[50]}\).

![Figure 28. Cyclomarin A, B, C, and D](image)

<table>
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<td>Cyclomarin C</td>
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<tr>
<td>Cyclomarin D</td>
<td>H</td>
<td>OH</td>
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</tr>
</tbody>
</table>

**Figure 28.** Cyclomarin A, B, C, and D
Rhizonin A and B are non-ribosomal cyclic heptapeptides with an unusual furanyl-alanine residue (Figure 29). They were first isolated from the fungus *Rhizopus microsporus*, though it was later determined that the true source was the endofungal bacterium *Burkholderia rhizoxina*. Both scaffolds are potent hepatotoxins\cite{51}.

**Figure 29.** Rhizonin A and B
Cordyheptapeptides A and B were isolated from the entomopathogenic fungus *Cordyceps* sp. BCC 1788 (Figure 30). Cyclic hexapeptides bearing three N-methyl groups and naturally occurring amino acid residues, Cordyheptapeptidide A exhibited antimalarial activity against the parasite *Plasmodium falciparum* K1 (IC$_{50}$ = 5.35 μM), though B showed no activity at 12 μM. Both peptides showed moderate cototoxicity against various human cancer cell lines with IC$_{50}$ values ranging from 0.2 to 2 μM[52][53].

The mushroom *Coriolus versicolor* was found to be the source of tetramethylated cyclic heptapeptide (−)-ternatin (Figure 31), which was found to suppress fat accumulation.

<table>
<thead>
<tr>
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<table>
<thead>
<tr>
<th>Cordyheptapeptide B</th>
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</thead>
<tbody>
<tr>
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</table>

**Figure 30.** Cordyheptapeptide A and B

**Figure 31.** (−)-Ternatin
against 3T3·L1 murine adipocytes (IC50 = 0.14 μg/mL), as well as having antimicrobial and antibacterial properties\[^{54}\]. The scaffold contains two interesting non-standard amino acid residues, (2R, 3R)-3-hydroxyleucine and D-allo-isoleucine, both of which were found to be essential for the activity of (-)-ternatin\[^{55}\].

### 1.2.5 Octapeptides

![Figure 32. RGD Octapeptide](image)

The Arg-Gly-Asp (RGD) unit of fibrinogen has been shown to inhibit the aggregation of human platelets. This three-amino acid sequence has been the focus of many combinatorial libraries. Nishino, et al, discovered an N-methylated octapeptide, cyclo(Arg-Sar-Asp-PhenylGly\(_2\)) (Figure 32), with potent inhibitory activity toward platelet aggregation (IC\(_{50}\) = 0.36), but no activity toward cell adhesion\[^{56}\].

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Bandunamide was isolated from *Streptomyces griseovariabilis bandungensis*. This tetramethylated octapeptide (Figure 33) contains three unusual residues that are methylsulfonylated at the β- and γ-carbons. This scaffold has exhibited antimicrobial properties against *Phytophthora drechsleri*, *Colletotrichum higginsiannum*, *Piricularia oxyzae*, and (IC$_{50}$ = 0.015, 0.0156, and 0.2 μg/mL, respectively)[57].

**Figure 33.** Bandunamide
1.2.6 Nonapeptides

Cylindrocyclin A was isolated from fungal extracts of *Cylindrocarpon* sp. A101-96 (Figure 34). This tetramethylated nonapeptide was found to be cytotoxic against six different cell lines (COLO-320, HL-60, L1210, Jurkat, MDA-MB-231, and MCF-7) with IC\(_{50}\) values from 11 to 53 μM\[58\].

A fungus derived from marine sponge *Halicondria japonica*, *Clonostachys rogersoniana* yielded the cyclic nonapeptides clonostachysin A and B (Figure 35). This highly methylated scaffold was found to have anti-

![Figure 34. Cylindrocyclin A](image)

![Figure 35. Clonostachysin A and B](image)
dinoflagellate activity against *Prorocentrum micans*, though they exhibited no antibacterial activity or other microalgae such as diatoms, cyanobacteria, and green algae[69].

1.2.7 Decapeptides

Isolated from a species of marine cyanobacterium *Oscillatoria*, the di-N-methylated decapeptide largamide H contains a number of unusual residues, including a β-amino acid, 3-amino-2,5-dihydroxydodecanoic acid (Adhda) (Figure 36). It is also a unique source of the amino acid 2-amino-5-(4'-methoxyphenyl)pentanoic acid (Amppa), and contains two residues of the non-standard 2,3-dehydro-2-aminobutanoic acid (Dab). Largamide H was tested for bioactivity against human colon cancer cell line HCT-116, but no activity was observed up to 250 μM[60].

![Figure 36. Largamide H](image)
1.2.8 Undecapeptides

Cyclosporine A is one of the most well-known \textit{N}-methylated cyclic peptides, and is a powerful immunosuppressant and anti-inflammatory agent approved for human use in 1983. It contains a novel amino acid residue, \((4R)\cdot4\cdot[\text{E}2\cdot\text{butenyl}]\cdot4\cdot\text{N-dimethyl-threonine (MeBmt)}\), which has been shown to be essential for the drug’s efficacy through SAR studies. Isolated from the fungus \textit{Toiyopocladium inflatum}, cyclosporine A is one of nine biologically active \textit{N}-methylated cyclic undecapeptide metabolites, named cyclosporine A through cyclosporine I\textsuperscript{[61]}. Cyclosporins A, B, C, D, and G differ only in the composition of a single amino acid, while cyclosporins E and I represent \textit{N}-demethylated congeners of A and D, respectively. It is interesting to note that, aside from the single D-valine present in cyclosporine H, the entire class of compounds shares the same peptide backbone configuration, and the \textit{N}-methylation pattern is highly conserved, with the exception of E and I (Figure 37). This is thought to preserve the intramolecular hydrogen bonding network and allow for the unexpectedly high degree of solubility, permeability, and bioavailability of this 11-amino acid cyclic peptide\textsuperscript{[62]}. Because of its high degree of complexity and well-documented biological properties, both in regards to activity and availability, many SAR studies and libraries based on the cyclosporine scaffold have been undertaken. PC-833, which has
Figure 37. Cyclosporine A-I and analogues

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<th>R₁</th>
<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
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been tested in phase III clinical trials under the name Valspodar, is a non-immunosuppressive cyclosporine A derivative that inhibits P-glycoprotein, a drug efflux pump responsible for multi-drug resistance\textsuperscript{[63]}. NIM-811 is a non-immunosuppressive cyclosporine A analogue that has been shown to exhibit cyclophilin inhibition without binding cyclophilin A. It inhibits mitochondrial permeability transistion, and is currently being investigated as a treatment for hepatitis C and other liver conditions\textsuperscript{[64]}. SDZ-IMM-125 is a cyclosporine derivative targeted at improving skin permeation of cyclosporine compounds. Strategic introduction of a polar side chain affords this compound a 16-fold increase in human skin penetration over cyclosporine A\textsuperscript{[65]}. SCY-635 is a cyclosporine based analogue that has high bioavailability and stability in human serum, is non-immunosuppressive, and exhibits potent suppression of hepatitis C virus replication \textit{in vitro} with an EC\textsubscript{50} of 0.10 μM\textsuperscript{[66]}. FR901459 is a cyclosporine-like cyclic peptide isolated from \textit{Stachybotrys chartarum} No. 19392. It was shown that FR901459 inhibits lymphocyte proliferation with approximately one third the potency of cyclosporine A, with IC\textsubscript{50} values of 26.8 and 9.9 ng/mL, respectively. These immunosuppressant compounds could be effective not only in preventing the rejection of organ transplants, but also in the treatment of autoimmune diseases such as uveitis, type II diabetes, and rheumatoid arthritis\textsuperscript{[67]}. 

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1.2.9 Dodecapeptides

The cyclic dodecapeptides known as omphalotins were isolated from the basidiomycete *Omphalotus olearius* (Figure 38). Omphalotin A exhibits selective activity against the phytopathogenic nematode *Meloidogyne incognita* ($LD_{90} = 0.76 \mu M$) with approximately four times higher potency than the commercially available nematicide ivermectin ($LD_{90} = 4.6 \mu M$)[68][69]. Omphalotins B through I all contain a novel tricyclic tryptophan derivative and exhibit nematicidal activity with $LD_{90}$ values ranging from 2 to 5 $\mu g/mL$[70].

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</tbody>
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**Figure 38.** Omphalotins A (top) and B-I (Bottom)
1.2.10 Tetradecapeptides

The ascomycete fungus *Myrothecium verrucaria* XZ04-18-2 yielded four hexa-N-methylated cyclic tetradecapeptides, verrucamides A-D (Figure 39). Verrucamides A-D have shown cytotoxic activity against *Staphylococcus aureus* with IC50 values of 3.59, 5.31, 4.45, and 9.09 μg/mL, respectively[71].

<table>
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<tr>
<td>D</td>
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</tbody>
</table>

*Figure 39.* Verrucamides A, B, C, and D
1.3 Synthetic Approaches to N-methylated Cyclic Peptides

1.3.1 Benoiton Method

One synthetic approach to generating N-methylated peptides is to employ amine protected N-methylated amino acids in the linear synthesis. In a method developed by Benoiton, et al, a strong base can be used to deprotonate the amine when base-stable protecting groups such as Cbz or Boc are used\[^{[72]}\]. The amine can then be methylated using a methylating agent such as methyl iodide (Scheme 1). Protecting groups, also base-stable, are required for any side chains prone to reaction with the base or methylating agents. The incorporation of a crown ether provides access to a variety of N-alkylated amino acids, though yields are low.

![Scheme 1. Synthesis of Boc-protected methylated amino acids](image)

1.3.2 Friedinger Method

A method for accessing Fmoc-protected N-methylated amino acids was developed by Friedinger, et al\[^{[73]}\]. First, an Fmoc-protected amino acid

![Scheme 2. Synthesis of Fmoc-protected methylated amino acids](image)
is combined with an aldehyde in an acid-catalyzed condensation reaction to yield an oxazolidinone (Scheme 2). The intermediate oxazolidinone can then be reduced to the N-methylated amino acid using triethylsilane and trifluoroacetic acid. This method results in minimal racemization (<0.1%), gives high yields (>70%), and is highly versatile, owing to the variety of both Fmoc-amino acids and aldehydes commercially available.

1.3.3 Miller Scanlan

Another approach to N-methylation of peptides involves direct methylation of the N-terminal residue of the resin-bound peptide. In a method developed by Miller and Scanlan, the free N-terminus is first

![Diagram of the methylation process involving a resin-bound peptide, a nitrobenzenesulfonyl (NBS) protecting group, collidine, DMF, DBU, and dimethyl sulfate.]
presence of a hindered, non-ionic guanidinium base, MTBD (Scheme 3). MTBD is critical for the selectivity of the reaction, as stronger bases yielded non-specific methylation of the peptide backbone and weaker bases gave poor yields. Removal of the NBS protecting group is then affected via nucleophilic substitution using β-mercaptoethanol and DBU in DMF. No racemization was observed; however, when methylating His(Tmt)-containing peptides, an impurity attributed to the methylation of the side chain and loss of the trityl group was observed.

1.3.4 Mitsunobu

Using the same NBS protection/deprotection protocols, the Mitsunobu reaction can be used to methylate terminal amines of resin-bound peptides[75]. Triphenylphosphine and diisopropylazodicarboxylate (DIAD) are used to convert the free amine of the peptide and methanol into the corresponding N-methylated amine (Scheme 4). Under Mitsunobu conditions, peptides undergo complete conversion to the corresponding N-methylated peptide with no observed racemization and
no methylation of protected histidine side chains. With a total reaction
time of only five minutes, Mitsunobu conditions are efficient and the
preferred methylation conditions for peptides containing His(Trt)
residues.

1.3.5 Bts Protecting group

The N-benzothiazole-2-sulfonyl (Bts) protecting group has been
used both for site-specific methylation (Scheme 5) and activation of amino
acids for use in couplings to sterically hindered residues. Use of the Bts

\[
\begin{align*}
\text{H}_2\text{N} & \text{C} = \text{O} \quad \text{H}_2\text{N} \quad \text{R}_1 \\
& \text{Bts-Cl} \quad \text{K}_2\text{CO}_3 \quad \text{MeI} \quad \text{CH}_3\text{CN} \quad \text{Bts} \quad \text{Me} \quad \text{K}_2\text{CO}_3 \quad \text{PhSH} \\
& \text{O} \quad \text{N} \quad \text{Bts} \quad \text{O} \quad \text{N} \quad \text{Me} \quad \text{Me} \\
\end{align*}
\]

**Scheme 5.** Site specific methylation reaction with Bts protecting group

The Bts protecting group adds another viable approach to methylation of the
terminal amine of a peptide chain. The Bts protecting group is installed
via the Bts-chloride and the corresponding amino acid using two-phase
conditions, giving 87-91% yield of the desired protected amino acid.

Methylation of the amino acid then precedes using the base K\textsubscript{2}CO\textsubscript{3} and

\[
\begin{align*}
\text{Bts} \quad \text{N} \quad \text{C} = \text{O} \quad \text{HN} \quad \text{Me} \\
& \text{Cl} \quad \text{Ot-Bu} \quad \text{Na}_2\text{CO}_3 \quad \text{NaHCO}_3 \quad \text{CH}_2\text{Cl}_2/\text{H}_2\text{O} \\
& \text{O} \quad \text{N} \quad \text{Me} \quad \text{N} \quad \text{Me} \\
\end{align*}
\]

**Scheme 6.** Bts protected amino acid chloride coupling reaction
methyl iodide. The Bts group can then be removed by thiophenol\textsuperscript{[76]}. The activating effect of the Bts protecting group on the amino acid chloride allows synthetic access to challenging β-branched couplings, such as N-methyl-valine (Scheme 6).

1.3.6 Sub-monomer synthesis

Zuckermann, et al, have reported a synthetic method for accessing N-methylated glycine residues. This approach involves assembling the glycine residue from two separate commercially available “submonomers”

\[
\begin{align*}
\text{H}_2\text{N}\text{R}_1\text{COOH} \xrightarrow{\text{DIC, DMF}} \text{Br-CH}_2\text{COOH} \rightarrow \text{Br-CH}_2\text{CONH}_2\text{R}_1 \xrightarrow{\text{NH}_2\text{Me}} \text{MeNCONH}_2\text{R}_1
\end{align*}
\]

Scheme 7. Submonomer synthesis of methyl glycine (Scheme 7). Bromoacetic acid is first coupled to the terminal amine using standard DIC coupling conditions. This is followed by the addition of a primary amine, which undergoes a substitution with the bromine, yielding the alkylated terminal amine. This synthetic route is limited by the use of glycine as the basic amino acid residue to be modified, due to the non-stereospecific reaction conditions. It does, however, allow access to a variety of alkylated amines\textsuperscript{[77]}. 
1.3.7 Coupling to N-methylated Terminal Amines

There is no general method for coupling to N-methylated amino acids that is efficient for all cases. Near complete conversion, percent yield >99%, is essential for solid phase peptide synthesis. Separation of truncation sequences and transposition sequences from the desired sequence is often impossible, even by HPLC. Resin-bound N-methylated secondary amines frequently exhibit lower reactivity than solution phase reactions, requiring longer reaction times. These slow reaction times lead to undesired side reactions, such as racemization or diketopiperazine (DKP) formation. To combat these problems, several approaches have been developed and can be used individually or in tandem to obtain the desired final sequence[78].

The coupling reagents BEMT (2-Bromo-3-ethyl-4-methyl thiazolium tetrafluoroborate) and BEP (2-bromo-1-ethyl-pyridinium tetrafluoroborate) have been shown to be very efficient for the coupling of N-alkylated amino acids (Figure 40). BEMT and BEP were found to be much more reactive than the commonly used BOP·Cl or PyBrOP, with coupling reactions 70-80% complete within two minutes. Due

Figure 40. Coupling agents
to the very short coupling times, racemization and side reactions were
minimized, with only 4.59% racemization observed. Addition of HOAT
further improved the yield by reducing racemization to 1.42%\cite{79}.

Amino acid halides have been employed in difficult couplings, such as
coupling to N-alkylated amines. However, prepared amino acid halides
have a limited shelf life, and are particularly unstable if the side chain
contains an acid-labile protecting group, making them impractical for use
in automated peptide synthesis. Bis(trichloromethyl) carbonate, BTC, has
been used to form N-alkyl amides by generating amino acid chlorides \textit{in situ}\cite{80}. Due to the acid lability of N-methylated peptide sequences, the
hyper-acid sensitive resin TCP (trityl chloride polystyrene) was used. For

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hplc_traces.png}
\caption{HPLC traces of coupling to terminal L·Me·Ile. A: racemic mixture of
Fmoc·L/D·Me·Val; B: Fmoc·D·Me·Val; C: Fmoc·L·Me·Val.}
\end{figure}
couplings, the resin was combined with a solution of DIPEA, while the Fmoc-amino acid was combined with BTC and collidine, which was added to the pretreated resin after one minute. Quantitative conversions were observed with reaction times ranging from 30 minutes to 3 hours. Racemization observed was minimal (0.7-2.8%), even when coupling to N-methylated β-branched amino acids such as N-Me-Val. When the HPLC traces of couplings of pure L and D-amino acids are compared to the racemic mixture, no contaminants due to racemization are observed (Figure 41)[81].
1.4 References


68. Thern, B., Rudolph, J., Jung, G. Total Synthesis of the Nematicidal Cyclododecapeptide Omphalotin A by Using Racemization-Free


Chapter 2: N-methylated Cyclic Peptide Library

2.1 Synthetic Approaches to Obtaining N-methylated Peptides

Very few N-methylated cyclic peptide natural products are per-methylated, or methylated at every amide bond. Most contain both methylated and non-methylated residues. We postulated that the combination of the stereochemistry of the backbone and the increased rigidity conferred by N-methylation were affording these macrocycles biologically significant advantages, and set out to investigate the complex interaction of conformation, stereochemistry, and N-methylation pattern.

Previously, the most common synthetic approach used to access N-methylated peptides involved methylating the desired residues individually in sequence as the peptide chain was elongated using standard solid phase peptide synthesis\[^1\]. This involved adding a protecting group to the N-terminus of the peptide then methylating the amine, followed by a deprotection step. This approach has very poor atom economy, and the subsequent coupling of the next amino acid to the methylated terminal amine is frequently challenging, especially with beta branched amino acids. This requires the use of more expensive coupling reagents, repetition of the coupling reaction, and increased coupling times, leading to increased yield of side products such as sequence truncations and diketopiperazines. Low percent yields of the methylation reaction, as
well as the subsequent coupling reaction, are incompatible with synthesis on the solid phase, which generally requires at least 99% yield. Because N-methylation increases the rigidity of the peptide backbone, highly N-methylated linear peptides are often challenging to cyclize, requiring long reaction times and giving low cyclization yields. Perhaps the most restrictive aspect of the in-sequence methylation approach is that it requires predetermined knowledge of methylation patterns, making it useful for synthesizing known natural product scaffolds, but not as useful for discovering new N-methyl variants of lead compound scaffolds.

An alternate approach to N-methylation involves generating the cyclic peptide scaffold, then subjecting the resin-bound scaffold to methylating conditions (Scheme 1). In this novel approach to synthesis of

Scheme 1. Methylation reaction.

N-methylated cyclic peptides, the cyclic hexapeptide sequence cyclo(Leu-D-Leu-Leu-Leu-Pro-Tyr) was selected as a test scaffold to screen for conditions that might afford regioselective N-methylation on the solid
phase. A single proline residue is known to promote efficient cyclization, and a tyrosine residue is used as the anchor to the resin as it is amenable to side-chain attachment to the linker. The scaffold cyclo(Leu·D-Leu·Leu·Leu·D-Pro·Tyr) was selected from a previous study of the conformational effects of stereochemistry on conformation, and was computationally predicted to have a single discrete conformation when in a low dielectric solvent\cite{2}. The N-methylation synthetic approach involved selection of a solvent system that facilitated the formation of hydrogen bonds within the scaffold. It was also necessary to select a base capable of deprotonating only those amide protons that were solvent exposed and therefore not involved in hydrogen bond, followed by introduction of a methylating agent to methylate all of the deprotonated positions.

THF was selected as a possible solvent because of its low dielectric constant. As the solvent is unable to hydrogen bond with the cyclic peptide scaffold, the hydrophilic moieties within the peptide are forced into intramolecular hydrogen bonds, as allowed by the conformation conferred by the stereochemistry of the backbone. Initially, n-butyl lithium was investigated for the base; however, low yields were observed when this base was used, and the reaction was slow, requiring 36 hours. Also, the use of pyrophoric compounds such as n-butyl lithium was not desirable. Lithium tert-butoxide (LiOtBu) was then employed to effect the
deprotonation of the backbone amides. With the test scaffold, this base gave near quantitative yields. Throughout the experiments, methyl iodide was selected as the methylating agent. Using LiO\textsubscript{t}Bu and methyl iodide, the solvent system was then optimized in order to obtain the highest selectivity and yield (Table 1). While screening reaction conditions for our model scaffold, we found that the choice of solvent for both the base treatment and the CH\textsubscript{3}I addition affected the extent of N-methylation along with the total number and identity of the products.

<table>
<thead>
<tr>
<th>Base</th>
<th>Solvent</th>
<th>temp (°C)</th>
<th>eq CH\textsubscript{3}I</th>
<th>Additive</th>
<th>% conversion</th>
<th>No. of N-methyl groups observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>P\textsubscript{4}-phosphazine (10 eq)</td>
<td>THF/THF</td>
<td>-78→25°</td>
<td>10</td>
<td>-</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>DBU (5 eq)</td>
<td>THF/THF</td>
<td>25</td>
<td>50</td>
<td>-</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>n-BuLi (15 eq)</td>
<td>THF/THF</td>
<td>-78→25°</td>
<td>50</td>
<td>-</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>n-BuLi (15 eq)</td>
<td>THF/THF</td>
<td>-78→25°</td>
<td>50</td>
<td>LiCl (20 eq)</td>
<td>66%</td>
<td>1, 2</td>
</tr>
<tr>
<td>LiO\textsubscript{t}Bu (10 eq)</td>
<td>DMSO/DMSO</td>
<td>25</td>
<td>10</td>
<td>-</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>LiO\textsubscript{t}Bu (10 eq)</td>
<td>DCM/DCM</td>
<td>25</td>
<td>10</td>
<td>-</td>
<td>38%</td>
<td>1, 2, 3,</td>
</tr>
<tr>
<td>LiO\textsubscript{t}Bu (10 eq)</td>
<td>THF/THF</td>
<td>25</td>
<td>10</td>
<td>-</td>
<td>67%</td>
<td>4, 5</td>
</tr>
<tr>
<td>LiO\textsubscript{t}Bu (10 eq)</td>
<td>THF/DMSO</td>
<td>25</td>
<td>10</td>
<td>-</td>
<td>94%</td>
<td>1, 2</td>
</tr>
<tr>
<td>LiO\textsubscript{t}Bu (10 eq)</td>
<td>THF+DMSO</td>
<td>25</td>
<td>10</td>
<td>-</td>
<td>95%</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1. Methylation reaction conditions.

observed. For example, the use of CH\textsubscript{2}Cl\textsubscript{2} for both reactions tended to yield products with fewer N-methyl groups, whereas the use of THF in both steps caused greater N-methyl incorporation but less selectivity than
the use of DMSO for the CH₃I addition. We subsequently selected a set of diastereomers that showed varying selectivity in the on-resin N-methylation reaction and then further increased the number of N-methyl products observed for each scaffold by varying the reaction conditions. By attenuating the selectivity of the N-methylation chemistry and using different solvent systems and stereoisomeric scaffolds, we were able to observe 48 distinct N-methyl products ranging from nonmethylated to permethylated peptides. Once the most effective solvent system was determined, differing concentrations of the base were examined in order to optimize the selectivity of the methylation reaction (Table 2).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
<th>Conc. of LiO⁻ Bu⁻ (M)</th>
<th>% of total product</th>
<th>No. of N-methyls</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>cyclo [Leu D-Leu Leu Leu D-Pro Tyr]</td>
<td>0.1</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td></td>
<td>0.5</td>
<td>93</td>
<td>6</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td></td>
<td>1.0</td>
<td>95</td>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>AcN⁻ H⁻ [Leu D-Leu Leu Leu D-Pro Tyr]</td>
<td>1.5⁺</td>
<td>99</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>5</td>
<td></td>
<td>CO₂⁻ All⁻</td>
<td>1.5</td>
<td>4</td>
<td>35</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>cyclo [D-Leu Leu Leu Leu D-Pro Tyr]</td>
<td>0.1</td>
<td>11</td>
<td>51</td>
<td>38</td>
<td></td>
<td></td>
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<td>7</td>
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<td>0.5</td>
<td>4</td>
<td>61</td>
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<td>8</td>
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<td>9</td>
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<td>1.5</td>
<td></td>
<td>98</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>AcN⁻ H⁻ [D-Leu Leu Leu Leu D-Pro Tyr]</td>
<td>CO₂⁻ All⁻</td>
<td>1.5</td>
<td>21</td>
<td>79</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2. Base concentration dependence of the methylation reaction.
Use of 0.1M LiOt·bu in THF for the basic deprotonation, followed by draining of the base solution and addition of methyl iodide in DMSO afforded nearly complete conversion of the model peptide to a single partially methylated regioisomer of the cyclic peptide, showing the highest degree of selectivity. That similar conditions have been reported previously for the on-resin peralkylation of linear peptides\cite{3} suggests that the selectivity we observed was due to the conformation and the cyclic nature of the starting peptide. Because there are 5 possible N-methylation positions, there are 32 possible methyl variants of the model cyclic peptide, but only a single regioisomer was observed. The specificity shown for this model scaffold was excellent, and the conditions here were used to methylate a larger stereoisomer library.
2.2 Scaffolds Synthesized

As α-carbon stereochemistry can have a significant impact on conformation in cyclic peptides, we examined the effect of stereochemistry on the selectivity of N- methylation by performing the methylation reaction on 32 stereoisomers based on the sequence cyclo(D/L-Leu·D/L-Leu·D/L-Leu·D/L-Pro·L-Tyr). Among the 29 diastereomers that cyclized efficiently on the solid phase, 6 sequences yielded specific N-methyl adducts with >95% selectivity when treated with the same on-resin N-methylation conditions described above. Three of these sequences yielded a trimethyl product (scaffolds 3, 19 and 27),

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Residue Number</th>
<th>&gt;95% Selective</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leu Leu Leu Leu Pro Tyr</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Leu Leu Leu Leu Pro Tyr</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Leu Leu Leu Leu Pro Tyr</td>
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<tr>
<td>4</td>
<td>Leu Leu Leu Leu Pro Tyr</td>
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<td>Leu Leu Leu Leu Pro Tyr</td>
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<td>6</td>
<td>Leu Leu Leu Leu Pro Tyr</td>
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<tr>
<td>32</td>
<td>Leu Leu Leu Leu Pro Tyr</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Stereochemistry of synthesized peptide scaffolds
and three yielded a tetramethyl product (scaffolds 2, 18 and 26). The remaining sequences yielded permethylated product (two sequences), returned starting material (four sequences), or gave mixtures of varying complexity (17 sequences) (Figure 1).

A pattern emerged in the relationship between stereochemistry and selectivity among the six highly selective scaffolds. The three trimethylated scaffolds had four contiguous residues (Tyr6-Leu1-Leu2-Leu3) identical with the consensus sequence cyclo(L-Leu-D-Leu·L-Leu·D/L-Leu·D/L-Pro·L-Tyr). The three tetramethylated scaffolds were identical at the same four contiguous residues but shared a different consensus sequence, cyclo(D-Leu·L-Leu·L-Leu·D/L·Leu·D/L·Leu·D/L-Pro·L-Tyr) (Figure 2). We chose two of the selective, partially N-methylated products for further study: the trimethyl variant of scaffold 19 and the tetramethyl variant of scaffold 18.
In order to test the sensitivity of the methylation reaction, attempts were made to obtain per-methylated 19. Repetition of the methylation reaction to the resin-bound peptide yielded no increase in degree of methylation, even when the reaction was repeated up to five times. In order to synthetically access the permethylated scaffold, the solvent system for the reaction was changed. Rather than draining the LiOtBu/THF solution before adding methyl iodide in DMSO, the resin was first incubated in the base solution. Then the methyl iodide/DMSO was added without draining. The DMSO disrupted intramolecular hydrogen bonding and, with the base still present, the remaining amides were deprotonated and methylated.

Intramolecular hydrogen bonding is thought to be responsible for the selectivity of the methylation reaction. To test the hypothesis that

![Graph](image)

**Figure 3.** H-D exchange studies for scaffolds 18 and 19 and their methyl variants
intramolecular hydrogen bonding exerts regiochemical control in the N-methylation reaction by protecting certain amide N-H groups from alkylation, we performed hydrogen-deuterium (H-D) exchange studies on 18 and 19 and their N-methylated derivatives (Figure 3). For both of the nonmethylated scaffolds, all five amide protons exchanged to deuterium very rapidly, within minutes. However, for the methylated scaffolds, the remaining amide protons did not exchange with solvent within the time of the experiment. Thus, the free amide N-H groups in both methylated scaffolds were either protected sterically from solvent or were involved in highly stable intramolecular hydrogen bonds in deuterated chloroform. In addition, N-methylation seemed to stabilize these hydrogen bonds relative to the nonmethylated precursors as the amides that were protected from exchange in the methylated species were among those that exchanged rapidly in the nonmethylated precursors.

We postulate that the amide deprotonation events are sequential and cooperative, such that deprotonation of the most exposed N-H limits the flexibility of the molecule and renders one or more of the remaining exposed amides more acidic. In other words, initial deprotonation of the most exposed N-H could shift the precursor’s conformational equilibrium toward the hydrogen-bonded conformation found in the product. This hypothesis is supported by a report showing that hydrogen bonding
protects the involved amide N·H from H·D exchange and simultaneously increases the exchange rate of the amide whose carbonyl is participating as the hydrogen-bond acceptor.

Using 2D NMR techniques (HMBC, HMQC, TOCSY, COSY and NOESY), we determined the pattern of N-methylation for 18 and 19. Compound 19 yielded exclusively the trimethyl adduct (19-NMe₃), in which residues D-leucine2, leucine3 and tyrosine6 are N-methylated. Diastereomer 18 yielded a single tetramethyl adduct, 18-NMe₄, in which residues D-leucine1, leucine2, leucine4, and tyrosine6 are N-methylated (Figure 4).

Thus, the transposition of only two stereocenters between 18 and 19 (leucine1 and leucine2) led to a complete shift in both the pattern and degree of N-methylation, highlighting the impact that small stereochemical differences can have on the global conformations of small cyclic peptides.
In order to show that the regiochemistry is an important factor for hydrogen bonding, a regioisomer of \textbf{19-NMe}_3 was synthesized using in-sequence methylation to control the methylation pattern. The alternative methylation pattern was designed to disrupt the intramolecular hydrogen bonding capacity of this scaffold (\textbf{19'-NMe}_3). H-D exchange studies were conducted for this regioisomer. Both remaining amide protons exchanged within minutes, indicating that they were exposed to the solvent and not involved in hydrogen bonds (Figure 5).

As predicted from H-D exchange data, the NMR solution structures of \textbf{18} and \textbf{19} in deuterated chloroform showed intramolecular hydrogen bonds between the nonmethylated amide N-H groups and backbone carbonyls. In \textbf{19}, the leucine1 and leucine4 residues each form two transannular hydrogen bonds with one another and are flanked by two $\beta$-turns. In \textbf{18}, a single hydrogen bond between the backbone carbonyl of D-
proline5 and leucine2 generates two opposing non-classical turns that result in a twisted geometry. In both scaffolds, all of the N-methyl amides are in the trans configuration, and the N-methyl groups point away from the ring into solution, results consistent with the hypothesis proposed (Figure 6).

In order to demonstrate the robustness of the N-methylation reaction to a variety of side chain functional groups, individual amino acid substitutions at positions 3 and 4 of scaffold 19 were synthesized. All reactive side chains were protected with acid-labile protecting groups. Hydrophobic side chains, such as alanine, yielded the expected trimethyl product. Other side chain functional groups lacking a free amine or amide were also unaffected by the N-methylation reaction, yielding the trimethyl adduct. Cysteine(trityl), glutamic acid(tBu), serine(OrBu), and tryptophan(Boc) all yielded single trimethyl products. However, for side chains bearing nitrogen-rich functional groups, methylation of the side chain was observed. Asparagine(trityl), glutamine(trityl), and lysine(Boc)
all yielded tetramethyl products. Histidine(trityl) yielded a mixture of trimethyl and tetramethyl, and methionine yielded a complex mixture (Table 3).

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Table 3. Results of N-methylation experiments performed on various side chain substitutions
2.3 Methods

2.3.1 Synthesis of N-methylated Peptides

The initial model peptide was synthesized starting with the allyl ester of fluorenlymethyloxycarbonyl chloride (Fmoc)-protected tyrosine linked as a silyl ether via the phenolic hydroxyl group to an all-alkyl silyl-tethered polystyrene resin according to published procedures\[2\]. Elongation of the peptide chain proceeded through standard fmoc solid phase peptide synthesis. After the addition of the final residue, deallylation and final Fmoc removal were performed simultaneously with a solution of 1 equivalents Pd(Ph\(_3\)P)_4 in THF containing 10% (v/v) piperidine for 3 hours. A chelating wash was performed to remove traces of Pd using 5% (v/v) sodium diethyldithiocarbamate in 5% (v/v) diisopropyl ethyl amine (DIPEA) in dimethylformamide (DMF), followed by a resin wash sequence. Cyclization was performed with 3 equivalents HATU, 3.2 equivalents HOAT, and 5 equivalents DIPEA in DMF for 3 hours, followed by resin washing.

All other peptides were synthesized with the allyl ester of (Fmoc)-protected tyrosine linked as an ether via the phenolic hydroxyl group to the 2-chlorotrityl chloride resin. The loading was performed using 2 equivalents of the protected tyrosine and 4 equivalents of cesium carbonate in dry DCM for 3 hours. This allowed for much faster resin
loading and cleavage from the resin, as the all-alkyl-silyl-tethered linker required 14 hours, and the cleavage from the resin was effected by use of hydrofluoric acid for 3 hours, as compared to the much shorter 3-hour loading time for the 2-chlorotrityl resin and the shorter and milder cleavage conditions of 1% trifluoroacetic acid (TFA) in DCM for 1 minute. For large-scale syntheses (>0.5 mmol, or 1 g resin), peptides were synthesized and cyclized using an automated peptide synthesizer (Prelude, Protein Technologies). For the 32-diastereomer library, a 96-well deep-well filter plate was used (Arcticwhite) with a manifold for solvent removal and an 8-channel trigger dispenser to dispense wash solvents and the deprotection solution. In general, couplings were performed using 4 equivalents Fmoc-protected amino acid, 3.8 equivalents HBTU (O-(benzotriazol-1-yl)·N,N,N,N′,N′-tetramethyluronium hexafluorophosphate and 6 equivalents N,N-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) (0.1 M in amino acid) for 1.5–3 hours. Fmoc deprotections were carried out with 2% diazabicycloundecene (DBU) in DMF for 15 minutes. After each coupling and deprotection step, the resin was washed with DMF (3×), dichloromethane (DCM) (3×) and DMF (3×). Peptides were either cleaved following cyclization or N-methylated and then cleaved. After evaporation, the peptide was taken up into acetonitrile (ACN) in H2O (3:1) and purified by reverse-phase preparative HPLC. For
peptides that contained side chain protecting groups, a final deprotection was performed using a solution of 5% (v/v) trimethylsilane in trifluoroacetic acid (TFA) for 1 h. The solution was evaporated, and the peptide was taken up into a 1:1 solution of water and ACN and either purified immediately or lyophilized and stored at −20 °C.

The following procedure was developed for on-resin N-methylation using 1 g of resin at a loading of ~0.13 mmol/g of peptide: A solution of lithium tert-butoxide (25 ml; 1.5 M) in dry THF was filtered through a 0.2-μ syringe filter and then added to the resin. After 30 min the base solution was drained and, without rinsing, a 10% (v/v) solution of methyl iodide in DMSO (25 ml) was added and the resin was agitated for 30 min. The resin was rinsed with water (1×), methanol (3×), DCM (3×), DMF (3×) and DCM (3×) and then was dried under vacuum. The cyclic peptide was cleaved, and the crude peptide was purified by reverse-phase HPLC on a C-18 10-μ 250 mm × 20 mm column using a typical gradient of 40% H2O in ACN (with 0.1% TFA) to 100% ACN (0.1% TFA) over 1 h. The resulting peptide was lyophilized.

In order to synthesize permethylated scaffolds, cyclic peptides were synthesized as above, and, without cleaving, the resin (0.1 g, 0.4 mmol/g) was dried in isolation with phosphorus pentoxide for 24 hours. A mixture of dry THF (3 ml) and dry DMSO (2.4 ml) was then added to the resin and
agitated with dry N2. Lithium tert-butoxide (32 mg, 10 equivalents) was added, and the mixture was agitated with N2 for 20 min. Six hundred microliters of CH3I was added via syringe, and the reaction was agitated with N2 for 20 min. The resin was filtered and washed multiple times with THF and DCM (Scheme 2).

Scheme 2. Synthetic scheme for solid phase synthesis and on-resin N-methylation of cyclo[Leu, Leu, Leu, Leu, Pro, Tyr]. X = all-alkyl diisopropyl silyl resin. Conditions: (a) 20% piperidine(v/v) in DMF; (b) Fmoc-aa-OH, HBTU, DIEA, DMF; (c) Pd(Ph3P)4, piperidine (10% v/v), THF; (d) PyBOP, HOAt, DIEA, DMF; (e) base, additive, CH3I (see Table 1). (f) 5% HF/py in THF.

2.3.2 H-D Exchange Studies

All NMR spectra for the H-D exchange studies were recorded at 297 K on a 600-MHz Varian Inova spectrometer equipped with a 5-mm
inverse detection probe. Spectra were referenced to residual solvent proton signals (1H 7.26 for deuterated chloroform). Peptides were dissolved in 665 μl deuterated chloroform at 6 mg ml⁻¹. Before the H·D exchange reaction was initiated, a spectrum was recorded (t = 0). The NMR tube was removed from the spectrometer, and a solution of 35 μl methanol monohydrate containing 10% trideuter-acetic deuteracid (CD₃CO₂D) was added by syringe to a final concentration of 5% (v/v) methanol monohydrate and 0.5% CD₃CO₂D. Upon addition of the deuterated solvents and quick agitation, a stopwatch was started and the NMR tube was returned to the spectrometer. Spectra were recorded using eight free induction decay transients for each time point at increasing intervals over a period of 16 h. H·D exchange rates were measured by integrating each exchangeable amide resonance separately and recording the ratio of its peak area versus the peak area for a downfield nonexchangeable C(α)H resonance.
2.4 Spectroscopic Data

**NMR spectroscopy and structure determination.** All NMR spectra were recorded at 297 K on a 600 MHz Varian Inova spectrometer equipped with a 5mm inverse detection probe. Spin multiplicity is described by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, and br = broad. Spectra were referenced to residual solvent proton and carbon signals (δ_H 7.26, δ_C 77.0 for CDCl₃). Spectra were processed and analyzed using MestReNova (Mestrelab) on a Macintosh G5 desktop computer.

Assignment of resonances for each amino acid residue was accomplished by employing ^1^H, ^1^C, gCOSY, HMQC and HMBC, TOCSY. Each residue was assigned using ^1^H chemical shifts and confirmed through COSY and HMQC crosspeaks. Assembly of these individual residues to form the final cyclized structure was accomplished by considering long range HMBC correlations from both α-protons and NH protons to adjacent carbonyl carbons, and α-proton to NH proton NOESY correlations between adjacent amino acid residues. NOESY correlations were documented for all available signals, and converted into inter-proton distances using the relaxation matrix analysis of proton 2D NOE spectra from the volume integrals of Nuclear Overhauser Enhancement Spectroscopy (NOESY) spectra[^4]. NOESY experiments were performed with mixing time of 90
ms, NOE build-up curves were constructed to verify that the NOEs were in the linear range, confirming that the initial rate approximation is valid\[5\].

Structure calculations were performed using CYANA[6]. on a Macintosh G5 desktop computer. The $\mathcal{J}$(NH-Hα) coupling constants were obtained from 1D spectra recorded with 64k complex points and a spectral width of 7804 Hz. The two $\mathcal{J}$(NH-Hα) couplings (Leu1: 9.49 Hz; Leu4: 9.15 Hz) were converted into dihedral restraints (+/- 15°) using the Karplus equation and input into CYANA. Upper and lower distance restraints were obtained by adding 10% to the calculated experimental distances for the upper limits and subtracting 10% of the calculated experimental distance for the lower limit. The final distance matrix was input into CYANA and a Molecular Dynamics (MD) simulation was performed to calculate 150 structures.
Figure 7. $^1$H-NMR Spectrum of 19 in CDCl$_3$. 
Figure 8. HMBC NMR Spectrum of 19 in CDCl₃.
Figure 9. Non-gradient HMQC NMR spectrum of 19 in CDCl₃
Figure 10. COSY NMR Spectrum of 19 in CDCl$_3$. 
Figure 11. TOCSY NMR Spectrum of 19 in CDCl₃.
Figure 12. NOESY NMR Spectrum of 19 in CDCl₃ (Mixing time = 70 ms)
Figure 13. $^1$HNMR Spectrum of 18 in CDCl$_3$. 
Figure 14. COSY NMR Spectrum of 18 in CDCl₃.
Figure 15. TOCSY NMR Spectrum of 18 in CDCl$_3$
Figure 16. HSQC NMR Spectrum of 18 in CDCl₃
Figure 17. HMBC NMR Spectrum of 18 in CDCl₃
Figure 18. NOESY NMR Spectrum of 18 in CDCl$_3$ (Mixing time = 90 ms)
Figure 19. HPLC/MS Data of 19 for different methylation reaction conditions.
Figure 20. HPLC/MS Data of crude product from resin cleavage of 18 for different methylation reaction conditions. Linear products marked NMe$_6$ indicate products in which the terminal residue was doubly methylated.
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<td>31.3, CH&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Tyr^6 α, D-Pro^5 CO</td>
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**Table 4.** NMR data for 19 acquired in CDCl₃
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<tr>
<th>Residue</th>
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<th>$\delta_\text{C}$, mult.</th>
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<th>HMBC</th>
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<tr>
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<td>-</td>
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<td>5.48</td>
<td>52.46, CH</td>
<td>Leu1 $\beta$</td>
<td>Leu1 CO, $\beta, \gamma$; Tyr CO</td>
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<td>Leu1 CO, $\alpha, \delta$2</td>
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<td>$\delta 2$</td>
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<td>D-Leu$^2$</td>
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<td>Leu3 $\alpha, \gamma$</td>
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<td>Leu3 $\beta, \gamma$, CO, NMe</td>
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</tbody>
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Table 5. NMR data for 18 acquired in CDCl₃
2.5 References


Chapter 3: Biological Effects of N-Methylation

3.1 Conformational Hypothesis of Cell Permeability

Backbone N-methylation is a hallmark of cyclic peptide natural products, and it seems to be correlated with increased membrane permeability in this class of compounds. In a recent study of a series of cyclic hexapeptides, Ovadia et al.\textsuperscript{[1]} demonstrate that both the number of N-methyl groups and, more importantly, their relative positions along the backbone can have a dramatic effect on cell permeability, although they did not explicitly investigate the role of conformation in determining permeability. We reasoned that the same conformational determinants that influence permeability in nonmethylated cyclic peptides could be used to impart selectivity by directing N-methylation to the most exposed N-H groups. Moreover, we postulated that the resulting N-methyl derivatives would have improved membrane permeability relative to the nonmethylated parent compounds.

Cyclic peptides tend to have molecular weights and polar group counts that place them outside most classical definitions of ‘drug-likeness’, challenging us to devise new rules for predicting pharmacokinetic properties (for example, oral absorption) for this class of compounds. N-methylation may serve to increase membrane permeability by allowing (or possibly stabilizing) intramolecular hydrogen bonding in the membrane-
associated state (Figure 1a). This hypothesis is consistent with previous reports studying the effect of conformation on membrane permeability for cyclic peptides, in which intramolecular hydrogen bonding is a key determinant of passive membrane diffusion in flexible molecules containing hydrogen-bond donors and acceptors. On the basis of this conformational hypothesis, we investigated whether intramolecular hydrogen bonding could be used to direct the N-methylation of a cyclic peptide scaffold on the solid phase (Figure 1b) and whether selective N-methylation could be used to improve the passive membrane permeability.
of the parent scaffolds. In general, the relative permeabilities predicted by our model are borne out by \textit{in vitro} and \textit{in vivo} pharmacokinetics studies.

Large cyclic peptide natural products, and macrocycles in general, may occupy ‘islands of bioactivity’ (a term coined by D. Hepworth and S. Liras of Pfizer) in chemical space that become increasingly sparse with increasing molecular mass. We postulate that many of these islands are populated with bioactive natural products whose properties have been optimized by the action of natural selection, although it is likely that many other islands remain undiscovered. The synthetic approaches described here may open these regions of chemical space up to the discovery of new macrocyclic scaffolds with drug-like oral bioavailability similar to \textbf{19-NMe$_3$}, a compound with a molecular mass of 755 Da that nonetheless achieves acceptable oral bioavailability.
3.2 Effects of N-Methylation on Permeability

The parallel artificial membrane permeability assay, or PAMPA, is often used to determine the passive membrane diffusion rates of drug candidates. PAMPA distinguishes passive membrane diffusion behavior from active-transport mechanisms\[^4\], and the resulting permeability values correlate well with *in vivo* absorption rates\[^5\] for drugs that diffuse by transcellular mechanisms. Results are measured as %T, which is calculated as the percentage of analyte entering the acceptor compartment after 12 h, where 100% T corresponds to equilibrium between donor and acceptor compartments.

We tested the passive membrane diffusion rates of scaffolds 18 and 19, their selectively N-methylated products, 18-NMe\(_4\) and 19-NMe\(_3\), and their permethylated derivatives 18-NMe\(_5\) and 19-NMe\(_5\) using PAMPA. For both scaffolds, the partially methylated derivatives were significantly more permeable than their nonmethylated precursors (Figure 2). Indeed, the PAMPA permeabilities of were comparable to that of the orally bioavailable drug propranolol. Notably, both permethylated derivatives were significantly less permeable by PAMPA than their partially methylated derivatives, with permeabilities as low as those of the nonmethylated precursors. This result suggested that each scaffold has an optimal number of N-methyl groups with respect to membrane
permeability, and that, at least for some small, conformationally constrained cyclic peptides, membrane permeability is not simply proportional to the number of N-methyl groups in the backbone.

To further test the hypothesis that conformation is the main determinant of membrane permeability, we synthesized a regioisomer of 19-NMe₃ to generate 19'-NMe₃ (Figure 3), whose passive membrane diffusion rate we investigated using PAMPA. Although 19'-NMe₃ differs from 19-NMe₃ by the placement of a only single N-methyl group, it is hypothesized to be less permeable because of its inability to form internal hydrogen bonds in a low-dielectric environment. In contrast to the

Figure 2. Permeabilities of nonmethylated, partially methylated, and permethylated scaffolds, as determined by PAMPA
compact $\beta$-turn structure of $\textbf{19-NMe}_3$, $\textbf{19'-NMe}_3$ has an irregular geometry in which one of the three $N$-methyl groups is in the cis amide configuration and both free amide $N$-H groups are exposed to solvent. This decrease in permeability was indeed observed (Figure 2).

Using less selective solvent systems for the $N$-methylation reaction, complex mixtures of various degrees of $N$-methylation were synthesized for four scaffolds. Without purification, PAMPA permeability measurements were made on the $N$-methyl product mixtures and the nonmethylated precursors. Independent control studies were performed on a set of pure compounds to verify that %T values from the product mixtures were the same as the %T values determined for the pure compounds. The results show that even among compounds with the same number of $N$-methyl groups (that is, regio- and stereoisomers), permeabilities vary greatly (Figure 4). The most permeable trimethylated compounds were more permeable than any of the permethylated species. All four scaffolds produced at least one $N$-methyl product with a PAMPA permeability ($\% T > 25\%$) comparable to that of many orally bioavailable drugs[26]. Thus, even among scaffolds that showed poor selectivity in the

Figure 3. $\textbf{19'-NMe}_3$ methylation pattern
on-resin N-methylation chemistry, N-methylated derivatives exist that are highly permeable, which indicates that selectivity in the on-resin N-

![Figure 4](image.png)

Figure 4. Plot of permeabilities measured vs. number of methyl groups for all regioisomers observed in each group

methylation reaction is associated with, but not a precondition for, excellent permeability. These compounds are accessible, in principle, using the type of site-specific N-methylation chemistry used to synthesize 19'-NMe₃.

The permeabilities of peptides 18, 19, and their N-methylated analogs were also examined in a cell-based Ralph Russ canine kidney (RRCK) permeability assay. This assay uses a stable population of Madin-Darby canine kidney (MDCK) cells that were selected by flow cytometry to have little or no functional P-gp, and therefore provide a good 104
measure of passive, transcellular permeability with minimal interference from active transport mechanisms associated with Pgp expression.

Compounds 18 and 19 showed relatively low cell permeability in RRCK cells, while their partially methylated derivatives were significantly (3 to 10-fold) more permeable than the corresponding nonmethylated precursors (Table 1). As in the PAMPA results, compound 19-NMe5 appears to be less permeable than the partially methylated species in the RRCK assay. However, 18-NMe5 was the most permeable among the compound 18 series in the RRCK assay, a result that contrasted with the PAMPA data showing 18-NMe5 to be less permeable than partially methylated 18-NMe4. All of the N-methylated compounds in this study had better cell permeability in the RRCK assay than the orally bioavailable drug CSA.

For compound 19, the same trend was observed in the RRCK permeability assay, in which 19-NMe3 was more permeable than both 19 and 19-NMe5. This outcome was probably due to the greater solvent exposure of hydrophilic carbonyl groups in the permethylated derivatives.

<table>
<thead>
<tr>
<th>compound</th>
<th>RRCK (x 10^6 cm/s)</th>
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<td>CSA</td>
<td>1.1</td>
</tr>
<tr>
<td>19</td>
<td>1.8</td>
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<tr>
<td>19-NMe3</td>
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<tr>
<td>19-NMe5</td>
<td>3.5</td>
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<td>18</td>
<td>0.5</td>
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<td>18-NMe4</td>
<td>4.1</td>
</tr>
<tr>
<td>18-NMe5</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Table 1. Permeabilities measured using RRCK cell line

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rather than being sequestered in hydrogen bonds in the partially methylated compounds. As none of the amide carbonyls in permethylated cyclic peptides can participate in intramolecular hydrogen bonding, we predict that these compounds are less permeable, in general, than those partially methylated derivatives that can achieve optimal internal hydrogen bonding. Because the synthetic strategy outlined here afforded preferential N-methylation of exposed N-H groups over those involved in internal hydrogen bonds, we postulate that these conditions can lead to methylated cyclic peptides with optimal or nearly optimal passive membrane permeability. In many cases, the most optimal conformation in terms of permeability may not be the predominant conformation selected by the N-methylation chemistry. However, given that hydrogen bonding is favorable to both permeability and selectivity, it is likely that the products of the on-resin N-methylation reaction will be among the most permeable of the many possible N-methyl variants.

**PAMPA assay to determine passive membrane diffusion rates: Methods**

A 96-well donor plate with 0.45µ hydrophobic Immobilon-P membrane supports (Millipore) and a 96-well Teflon acceptor plate were used. The acceptor plate was prepared by adding 300 µL of 5% DMSO/PBS to each well. Two solutions were necessary for the donor plate. 10 µM solutions of the cyclic peptides were prepared in 5%
DMSO/PBS buffer, and a 1% (w/v) solution of lecithin in dodecane was prepared and sonicated before use. 5 µL of the dodecane lecithin solution was carefully applied to the membrane supports in the wells of the donor plate, being careful not to touch the pipet tip to the membrane. Without allowing this solution to evaporate, 150 µL of the peptide solutions were added to the donor wells. The donor plate was then placed on top of the acceptor plate so that the artificial membrane was in contact with the buffer solution below. A lid was placed on the donor well, and the system was covered with a glass evaporating dish and left overnight on a benchtop at room temperature. A wet paper towel was placed on the inside of the chamber to prevent evaporation.

Acceptor and donor well concentrations were measured by LCMS (Finnigan LTQ) using selected ion monitoring (SIM) mode. An internal standard of H$_2$N-tyrosine-O(allyl ester) was run with each sample, so that ratios of peak areas of compound to standard from the TIC detector could be used to measure relative concentrations. Donor well starting ratios of compound to standard were used as the initial concentration.

Stocks (1 mM) of the LCMS standard and each test compound were prepared in DMSO. From these, 10 µM solutions of the test compounds were prepared in 5% DMSO/PBS. A 1:2 dilution of the 10 µM stock was prepared in order to represent the theoretical equilibrium concentration of
the donor and acceptor wells. Next, a solution was prepared containing 90 µL of the theoretical equilibrium mixture, 10µL of the 10 µM standard, and 90 µL of methanol in a 200 µL LCMS vial insert. 20 µL of this solution was injected into the LCMS. Ratios of analyte-to-standard peak areas were then calculated as initial donor well concentrations. For the acceptor wells, a solution was prepared by mixing 90µL taken from the acceptor well after 12 hours, 10µL standard, and 90µL methanol. Multiple time points of the acceptor wells were taken in this same manner (90 µL of acceptor well, 10 µL of 10 µM standard, 90µL methanol, 20 µL injection onto LCMS). Each cyclic peptide was run in triplicate and averages were taken to obtain the final %T values.
3.3 Bioactivity Assays

In addition to the cell permeability studies, the compound series based on 18 and 19 was tested *in vitro* for stability in human plasma and in liver microsomes from rats and humans. These assays provide a general measure of the stability of compounds toward degradation by proteolytic and cytochrome P450 enzymes, respectively. The stability of all of the cyclic peptides studied in human plasma (terminal elimination \((t1/2) >360\) min) is consistent with the stabilizing effect of cyclization (and possibly the presence of D-residues) toward proteolytic degradation. In human liver microsomal stability assessments, compounds 18 and 19 and their methylated variants were less stable than CSA, as reflected in the

<table>
<thead>
<tr>
<th>compound</th>
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<th>RLM Clint (µg/min/mg)</th>
<th>Human plasma stab ((t1/2,) min)</th>
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<tr>
<td>CSA</td>
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<td>62.6</td>
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*Table 2.* Stability results for nonmethylated, partially methylated, and permethylated scaffolds 18 and 19 in Human Liver Microsomes (HLM), Rat Liver Microsomes (RLM), and human plasma.
higher intrinsic clearance (CL\text{int}) values (Table 2). Further, the partially methylated compound \textbf{19-NMe}_3 had greater microsomal stability than either its nonmethylated or permethylated derivative, whereas partially methylated \textbf{18-NMe}_4 was significantly less stable than either \textbf{18} or \textbf{18-NMe}_5. Overall, for both series, the greater stability in rat microsomes compared to human liver microsomes suggests species-specific differences in metabolism. Remarkably, compounds \textbf{18} and \textbf{19} had very different microsomal clearance rates even though they have the same sequence and molecular weight and differ only by the relative configurations at two stereocenters. Thus, conformation not only has a large role in determining passive cell permeability in cyclic peptides but also has a significant impact on their stability toward oxidative metabolism.

The \textit{in vivo} pharmacokinetics of \textbf{19-NMe}_3 in male Wistar-Han rats are summarized in Table 3. The intravenous pharmacokinetics of \textbf{19-NMe}_3 in rats are characterized by a low peritoneal clearance (~4.5 ml min\(^{-1}\) per

<table>
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<th>p.o. administration</th>
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<tr>
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<td>CL (mL/min/kg)</td>
<td>Vdss (L/kg)</td>
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<tr>
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</tr>
<tr>
<td>CSA</td>
<td>3.5</td>
<td>1.2</td>
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\textbf{Table 3.} Pharmacokinetic results for \textbf{19-NMe}_3
kg body weight) and a moderate volume of distribution ($V_{dss}$) (1.1 liters per kg body weight), leading to a $t_{1/2}$ of 2.8 h. The absolute oral bioavailability ($F$) of $19\cdot\text{NMe}_3$ was determined to be $\sim28\%$, a value remarkably similar to that reported for CSA. CSA and $19\cdot\text{NMe}_3$ also had very similar area under the curve (AUC) and maximum observed concentration ($C_{\text{max}}$) values following the same oral dose of 10 mg kg$^{-1}$.

It is worth noting that the scaffold and N-methylation pattern found in $19\cdot\text{NMe}_3$ is similar to that identified by Biron et al. in their N-methyl scan of the Veber-Hirschmann somatostatin analog cyclo[Pro–Phe–D-Trp–Lys–Thr–Phe]$^4$. In their study, an extensive N-methyl scan led to 30 analogs, of which a subset cyclized efficiently to yield the desired N-methyl cyclic peptide. Of the seven N-methyl variants whose affinity toward the somatostatin receptor was similar to that of the wild-type sequence, only the parent sequence and a single trimethyl variant, cyclo(Pro–Phe–D-Trp(N-methyl)–Lys(N-methyl)–Thr–Phe(N-methyl)), showed significant $in\ vivo$ uptake. Although the relative placement of the conformation-determining D-tryptophan and proline residues in this trimethylated version of the Veber-Hirschmann peptide differs from that in $19\cdot\text{NMe}_3$, both peptides adopt similar backbone structures in which the D residue and the proline form two opposing $\beta$ turns with the nonmethylated amides participating in transannular hydrogen bonds.
This conformation, in which the positioning of the two D residues templates the formation of two opposing β turns, is most likely the key determinant of both the N-methylation regiochemistry in compound 19 and the membrane permeability of its trimethyl adduct. The reported oral bioavailability of this peptide is 9%, a value comparable to 28% in 19-NMe₃.

Although the strategy reported here may help identify new membrane permeable scaffolds, its ability to confer bioavailability to peptide sequences with known bioactivity is limited. In many cases, N-methylation is likely to diminish biological activity by either directly blocking interactions of the backbone with its receptor or driving the peptide into an inactive conformation. However, varying both stereochemistry and N-methyl placement for a given sequence may yield unique scaffolds that retain biological activity while showing improved permeability.

Cell-based phenotypic assays were performed on the entire library of nonmethylated and methylated scaffolds. In these assays, the Hoechst stain was used to measure the total number of living cells in each well. Edu (5-ethynyl-2′-deoxyuridine) was used to stain cells undergoing DNA synthesis, and PH3 antibody staining was used to determine the number of cells in mitosis. These numbers were then compared to the control well,
which contained only DMSO (Figure 5). The nonmethylated scaffolds 1-32, shown in green, have a much lower degree of variation from the control well; however, the methylated scaffolds (purple) showed a higher amount of phenotypic diversity. The most potent methylated scaffold, 27 (highlighted in red), was highly cytotoxic and showed the greatest difference in overall cell growth between the nonmethylated and methylated scaffold. Another phenotype was observed for scaffold 3. Here a relatively high incorporation of the Edu stain combined with a low PH3 antibody stain indicates that the cells are in S-phase arrest. Of the most selective scaffolds for methylation, 6 and 27 were the most active. Scaffold 6 was most selective for the permethyl variant, 6-NMe₅, while 27 was selective for a single trimethyl variant, 27-NMe₃. The cell staining for these compounds is shown in Figure 6. For both 6 and 27, the nonmethylated scaffold does not vary greatly from the DMSO control.

Figure 5. Normalized cell staining results
However, for the methylated scaffolds, definite phenotypes are observed. For scaffold 6, very few cells incorporate the Edu stain, and the overall number of living cells indicated by the Hoechst stain does not change appreciably. The number of cells observed undergoing mitosis is still high, indicating a mitotic arrest phenotype. Scaffold 27, however, shows
an overall decrease in the number of living cells, indicating general cytotoxicity.

For these assays, the methylated products were not purified, so the effective concentrations of each methylated product varied for each starting scaffold, dependent on the methylation chemistry. For highly selective scaffolds, the concentrations of the primary product will be highest. For scaffolds that are not selective, several methyl variants may be present in very low concentrations. While the most potent lead compounds from highly selective methylation reactions are easily determined, the methyl variants resulting from lower selectivity reactions will have lower abundance and be harder to detect. Purification of these scaffolds and further assays can be used to characterize these low abundance, yet possibly highly active, methylation products.
3.4 References


References


61. Rehman, H., Ramshesh, V. K., Theruvath, T. P., Kim, I., Currin, R. T., Giri, S., Lemasters, J. J., Zhong, Z. NIM 811(N-Methyl-L-4-isoleucine Cyclosporine), a Mitochondrial Permeability Transition Inhibitor,


