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N-Methylation of Peptides: A New Perspective in Medicinal Chemistry

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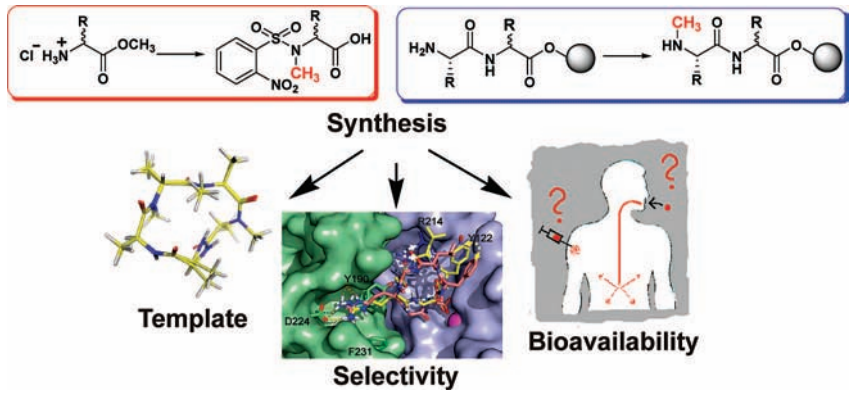

The potential of peptides as drug candidates is limited by their poor pharmacokinetic properties. Many peptides have a short half-life *in vivo* and a lack of oral availability. Inspired by the excellent pharmacokinetic profile of cyclosporine, a natural, multiply N-methylated cyclic peptide, we envisioned multiple N-methylation as a promising way to rationally improve key pharmacokinetic characteristics. In this Account, we summarize our efforts toward modulating the properties of peptides by multiple N-methylation.

As a first step, we simplified the synthesis of N-methylated amino acids in solution, by employing very mild conditions that could be tolerated by the diverse protecting groups required when working with naturally occurring amino acids. We also report the rapid and inexpensive syntheses of N-methylated peptides on a solid support; this facilitated the N-methyl scanning of bioactive peptides. Because of a lack of information regarding the conformational behavior of multiply N-methylated peptides, a complete library of N-methylated cyclic alanine pentapeptides was synthesized. The library provided valuable insight into the conformational modulation of cyclic peptides by N-methylation. This information is extremely valuable for the design of bioactive peptides and spatial screening of cyclic N-methylated peptides.

To demonstrate the applicability of N-methylation to highly active but poorly bioavailable peptides, we performed a full N-methyl scan of the cyclopeptidic somatostatin analog cyclo(-PFwKTF-), known as the Veber–Hirschmann peptide. We show here for the first time that the simple approach of multiple N-methylation can drastically improve the metabolic stability and intestinal permeability of peptides, for example, resulting in 10% oral bioavailability for a tri-N-methylated Veber–Hirschmann peptide analog. In addition, we also describe a designed approach to N-methylated peptide library synthesis, which can accelerate the screening of N-methylated bioactive peptides. Finally, we find that multiple N-methylation of a cyclic hexapeptide integrin antagonist of GPIIb-IIIa (αIIbβ3 integrin), cyclo(-GRGDfL-), increases the selectivity of this peptide toward different integrin subtypes. This result demonstrates the utility of multiple N-methylation in elucidating the bioactive conformation of peptides.

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Introduction

Peptides have found tremendous attention in diverse aspects of science ranging from rational drug design[1] to nanomaterials.[2] These diverse applications are due to their distinctive properties, such as ease of synthesis and characterization, introduction of chemical diversity by simple amino acid substitution, and modulation of 3D structure by chemical modification. The application of peptides as drugs stems from their key role in many signal transduction pathways, which makes them an attractive avenue to target diseases. Despite the high activity and receptor selectivity of naturally occurring bioactive peptides (or active protein fragments), they have distinct disadvantages for practical application in medicine, such as short half-life *in vivo* and lack of oral availability. The initial step in drug research of peptides is usually simplification (e.g., reduction in size), followed by peptidomimetic approaches to ensure metabolic stability, with the final goal of an orally available, highly active, and selective drug. Whereas the preliminary steps can be done in a rational way with relatively high probability of success, the final, crucial step of conversion from peptide into a drug is often more problematic.

Not all of the amino acids in a peptide sequence are essential to achieve the biological effect. The initial identification of the "bioactive sequence", the minimal sequence[3] required to achieve the biological activity, is often done by alanine scanning. This is the systematic substitution of each amino acid by alanine to identify the key residues, that is, those whose substitution results in reduced activity. The next important factor is the conformation of the peptide. In the majority of such peptides, a major obstacle in the study of the "bioactive sequence" is intrinsic flexibility. Thus, the active sequence must be rigidified in a defined conformation in order to achieve the desired activity and selectivity. Reduction of conformational space can be achieved by cyclization, resulting in highly active and selective derivatives when the bioactive conformation is matched.[4] This search of matching is done by "spatial screening".[5] Another major problem in developing peptidic drugs is their enzymatic degradation *in vivo*, which eventually results in the lowering of the pharmacokinetic profile (half-life, bioavailability, etc.). Medicinal chemists have developed an array of strategies over the years to confront this problem, such as incorporating peptide bond isosters,[6] peptoids,[7] retro-inverso peptides,[8] and peptidomimetics.[9] Although these strategies have elegant properties of their own, they demand careful design with challenging syntheses.

Here we envisage the minimalist approach of N-methylation to overcome various obstacles of peptides as a "rational" way toward drug development. Mono-N-methylation has been used for years to change phamacological properties of peptides.[10] However, due to difficulties in synthesis[11] and the expectation of losing activity, multiple N-methylation has seldom been used.[12] Although, there are prominent examples of multiply N-methylated natural cyclic peptides,[13] cyclosporine, omphalotin, etc. (Figure 1), with remarkable biological and pharmacological profile. Cyclosporine A, with its seven N-methylated peptide bonds, violates all the Lipinski rules for oral availability[14] but is marketed as an orally available (oral bioavailability 28% ± 18%) immunosuppressive drug. Thus, in recent years, we have introduced multiple N-methylation into cyclic peptides to characterize the versatile properties of this modification. We have developed an efficient and practical synthesis of N-methylated amino acids in solution[15] and on solid phase[16] and showed that multiple N-methylation not only can dramatically improve the receptor subtype selectivity[17] but also can confer oral bioavailability.[18] Here, we summarize the synthesis, conformational behavior,[19,20] pharmacokinetic properties and modulation of bioactivity by multiple N-methylation of cyclic peptides.

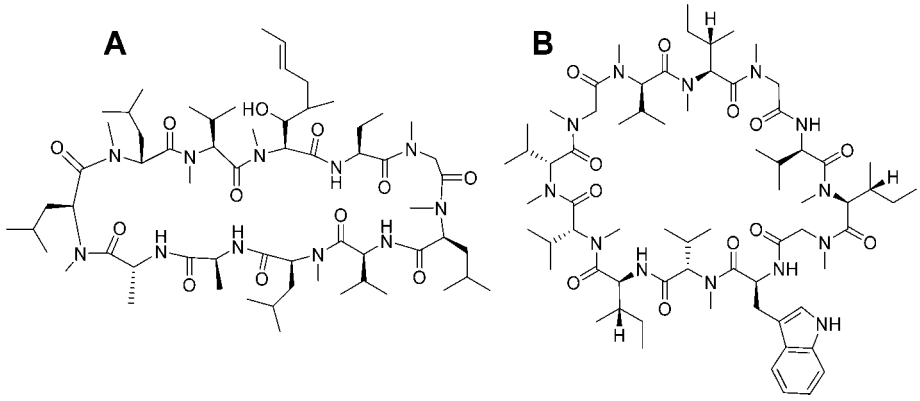


FIGURE 1. Naturally occurring multiply N-methylated cyclic peptides: (A) cyclosporine A and (B) omphalotine.

Synthesis of N-Methylated Peptides: Problems and Solutions

Over the years, multiply N-methylated peptides have failed to attract the attention of medicinal chemists due to various disadvantages encountered in their synthesis. First, a general approach to the synthesis of the N-methylated amino acids is a challenging task, and this is followed by the difficult coupling of the preceding amino acids to the sterically hindered N-methylated site. Synthesis of N-methylated peptides and cyclic peptides was revolutionized after the groundbreaking total synthesis of cyclosporin by Wenger.[21] The synthesis was carried out in solution using Boc chemistry, and owing to the fortunate lack of diversely functionalized amino acids in cyclosporine, the difficult couplings on the N-methylated terminus could be carried out by the formation of reactive acid chloride. In solid phase peptide synthesis, easy and fast coupling of N-methylated amino acids is achieved by using bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl),[22] 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt),[23] and bis-(trichloromethyl)carbonate (BTC).[24] We observed that, although expensive, difficult couplings with HATU/HOAt using double coupling are more efficient than using BTC and BOP-Cl. Although BTC is an effective reagent, it demands strict anhydrous conditions (alteration results in cleavage from the resin) and higher equivalents of base, which might lead to racemization.

We prefer solid phase synthesis for all linear precursor peptides. The sequence of amino acids during peptide synthesis and subsequent cyclization is crucial both for averting side reactions and for efficient cyclization. A common problem encountered in the solid phase synthesis of N-methylated peptides is the cleavage from the resin by the formation of diketopiperazine due to the higher population of cis peptide bond (of the tertiary amide bond) when an N-methylated amino acid or proline are loaded to the resin.[11] The cyclization yield of N-methylated peptides is strongly dependent on the linear sequence. We always perform the cyclizations in solution under high dilution conditions (0.1−0.3 mM), because cyclization on solid phase is often accompanied by cyclodimerizations due to the relatively high concentration of peptide on the resin. Linear peptides exhibit a large number of conformations in solution; hence, any preferred conformation that brings the C- and N-termini in close proximity enhances the cyclization yield. We encountered several instances where the linear peptide completely failed to cyclize owing to the lack of a preferred conformation in solution: for example, a somatostatin analog cyclo(-P^{6}F^{7}Mew^{8}MeK^{9}T^{10}MeF^{11}-) (small single-letter code denotes D-amino acid) exhibits a βII′-turn around D-Trp^8 and Lys^9 and a βVI-turn around Phe^{11} and Pro^6, and we could achieve efficient cyclization by using MeLys^9 as the C-terminus and Thr^{10} as the N-terminus. On the other hand, when Pro^6 and Phe^7 were taken as the C- and N-termini, respectively, the peptide completely failed to cyclize despite very high yield of the linear peptide (Figure 2). Thus, termini for efficient peptide cyclizations should be chosen in a way that results in the closure of a turn; preferably a βII/II′-turn.

Synthesis of N-Methylated Amino Acids and Peptides

Solution Synthesis of N-Methylated Amino Acids. When the goal is the synthesis of libraries of N-methylated peptides, it is preferable to use N-methylated amino acids directly as building blocks. Despite a plethora of available methods,[25] the commercially available N-methylated amino acids are still very expensive. Whereas amino acids with aliphatic side chains and without functional groups are best synthesized by the Freidinger method (via the reductive ring opening of the 5-oxazolidinone using TFA and triethylsilane),[26] the side chains of functionalized amino acids may react under these conditions. Hence, we developed an improved method yielding enantiopure N-methylated amino acids quantitatively to be used directly in Fmoc solid phase peptide synthesis.

The most efficient method for the site-selective N-methylation of peptides to date was developed by Fukuyama[27] and Miller.[28] This is a three-step procedure involving amine activation by protection with an *o*-nitrobenzenesulfonyl group (*o*-NBS), followed by alkylation and deprotection of the *o*-NBS group on a solid support (Scheme 1A). However, the major drawback of the procedure is that only small quantities of N-methylated amino acids can be prepared on the *solid phase*. Therefore, for the large-scale synthesis of N-methylated amino acids in *solution*, our strategy was to modify this procedure as follows:

- substitution of the expensive base MTBD (7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene) by a structurally similar inexpensive base DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and dimethyl sulfate as the methylating agent (Scheme 1B).
- masking the carboxyl group of the amino acid by a methyl ester for the synthesis of *N*-methyl-*o*-NBS-Xaa methyl esters.

The racemization-free saponification of the N-methyl-*o*-NBS-Xaa methyl esters to N-methyl-*o*-NBS-Xaa was done by a S_N2 mechanism, because N-methylated amino acids are infamous for undergoing racemization by base-mediated saponification due to the absence of an amide proton.[29] How-

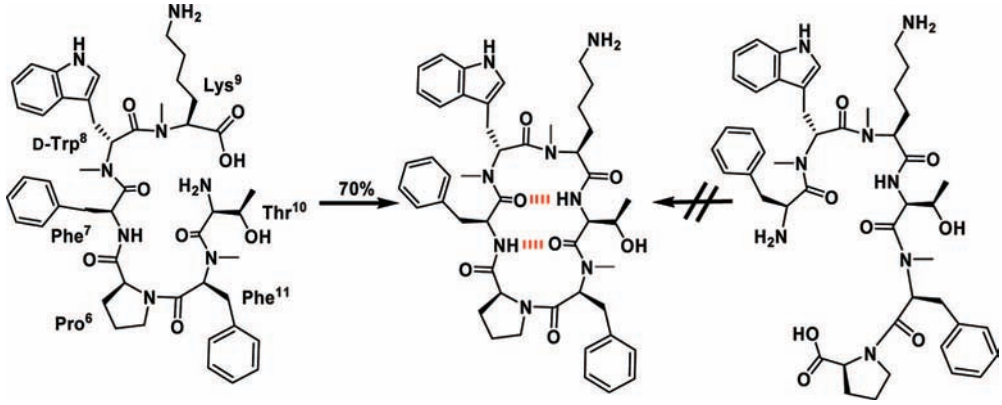
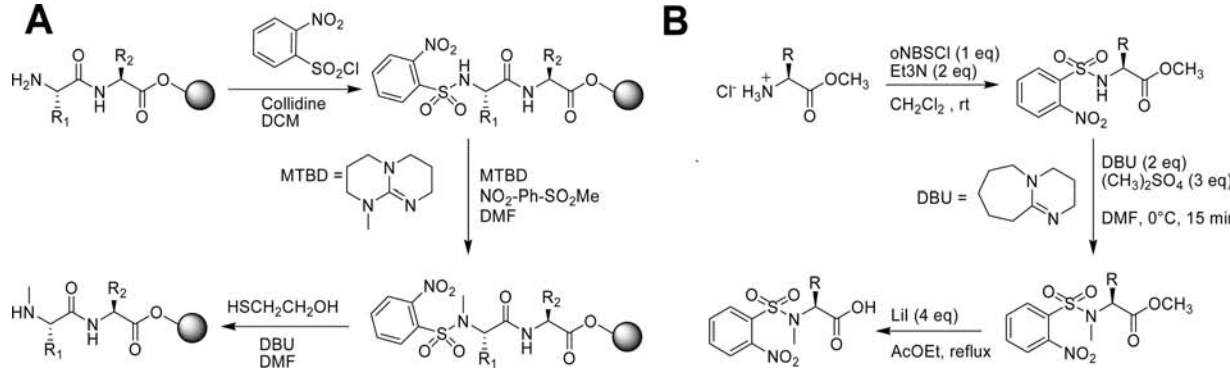


FIGURE 2. Choice of the preferred cyclization site in a linear peptide, TMeFPFMewMeK. The yield of the cyclized peptide is given.

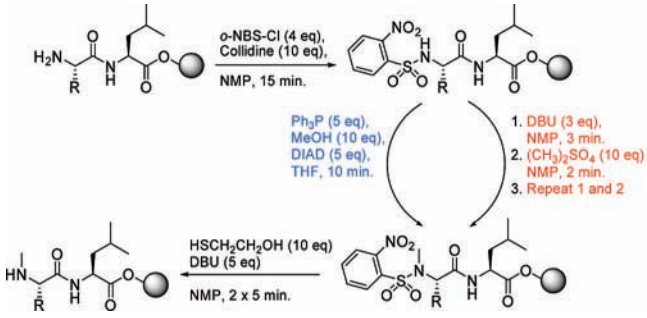
SCHEME 1. The Site Selective N-Methylation (A) on solid support and (B) Synthesis of N-Methyl-*o*-NBS-Amino Acids[15]



ever, LiI in refluxing pyridine[30] yielded a mixture of products (including cleavage of *N*-methyl groups due to nonselective attack of iodine), but the reaction worked well by using refluxing ethyl acetate for 16 h, yielding the corresponding enantiopure *N*-methyl-*o*-NBS-Xaa in quantitative yields to be used directly for solid phase peptide synthesis (SPPS).

Optimized Solid Phase Synthesis. When small amounts of amino acids are needed, for example, during N-methyl scanning of bioactive peptides, the simplest strategy is the direct N-methylation of the desired amino acid on the solid support during the peptide synthesis. The Miller and Scanlan procedure[28] was chosen, and optimization was done using DBU as base and the more polar NMP (*N*-methylpyrrolidone) as a solvent to reduce the total time (Scheme 2).

SCHEME 2. On-Resin Synthesis of *N*-Methyl-Xaa Dipeptides[16]



The optimization of the *o*-NBS protection step revealed completion of the reaction without any racemization in the surprisingly short time of 15 min in the polar NMP (*N*-methylpyrrolidone) using 4 and 10 equiv each of *o*-NBS-Cl and collidine, respectively, whereas reactions in THF (>2 h) or DCM (~1 h) required much longer reaction times.

The subsequent alkylation step in the original procedure, using MTBD in DMF is completed in 30 min. However, when 3 equiv of the less expensive DBU and 10 equiv of dimethylsulfate in NMP were used, the reaction was complete in only 5 min. This method was efficient for all the amino acids investigated, yielding products with >99% purity. The only exception was His(Trt), which showed N-methylation of the side chain with the unexpected loss of the trityl protecting group. To overcome this problem, the Mitsunobu procedure was used, although it requires the change of solvent from NMP to THF.[31] N-Methylation of the resin bound N^{α}-*o*-NBS-dipeptide is performed with 5 equiv of triphenylphosphine, 10 equiv of methanol, and 5 equiv of diisopropyl azodicarboxylate (DIAD) in THF. Monitoring the reaction over time revealed that only 10 min is required for the completion of the reaction, and hence it can be employed as a very fast and efficient method for N-methylation of peptides on a solid support. It is worth mentioning that this optimized method of N-methylation is

also efficient for the introduction of functionalized larger alkyl groups into peptides.[32]

The removal of the *o*-NBS protection is achieved by the formation of a Meisenheimer complex by reaction of the resin bound dipeptide with 5 equiv of DBU and 10 equiv of mercaptoethanol and was optimized to be complete in 5 min, compared with 30 min in the original procedure. Thus, we are now equipped with fast and highly efficient methods to prepare large amounts N-methylated amino acids and peptides to create libraries of multiply N-methylated peptides.

Conformational Impact of Multiple N-Methylation

Spatial Screening. The conformation of cyclic peptides of smaller ring size is mainly dictated by the pattern of chirality (D- or L-) of the amino acids in the peptide sequence.[20] Hence, peptides that consist of only alanine with a fixed pattern of chirality can be used as template structures for designing bioactive peptides, where alanine is replaced with appropriate amino acids (pharmacophores), barring glycine and proline. N-Methylation introduces another dimension to this "spatial screening"[5] (Figure 3) owing to the remarkable property of conformational modulation. N-Methylation facilitates the occurrence of a cis peptide bond and blocks potential hydrogen bonds, resulting in a long-range impact, especially on the backbone conformation of cyclic peptides.[33]

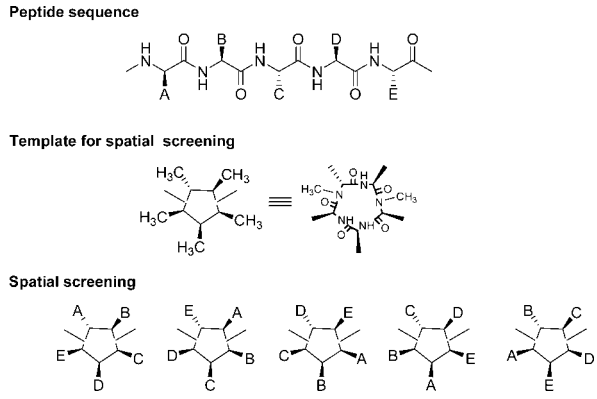


FIGURE 3. A peptide with pharmacophoric groups A, B, C, D, and E can be screened for the spatial orientation in the bioactive conformation by the synthesis of the five position-shifted cyclic isomers. In the absence of N-methylation, the five isomers would have identical constitution but present pharmacophores differently. In this example, however, the five di-N-methylated peptides with shifted N-methylated peptide bonds are constitutional isomers.

To elucidate the impact of N-methylation on the backbone of cyclic peptides, we synthesized a library of 30 N-methylated peptides, based on cyclo(-D-Ala-L-Ala_4-) (Figure 4). NMR analysis of these peptides displayed various populations of major and minor conformers slowly interconverting on the NMR time scale.[19] To serve as templates for rational drug design, we were interested only in those peptides that exhibited a preferred conformation (>80% population of a single conformer). It should be noted that in these small cyclic peptides, the conformation is dictated primarily by the steric interactions of the bulky $-CH_3$ groups (α-methyl or N-methyl), rather than the internal hydrogen bonds, which have been overemphasized over the years in stabilizing cyclic peptide conformation.

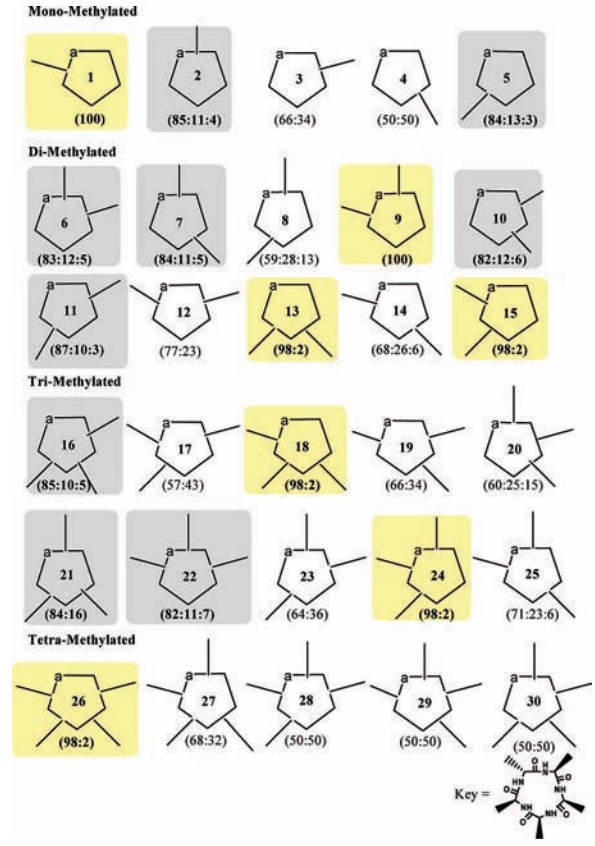


FIGURE 4. The library of N-methylated cyclic alanine peptides. Numbers in parentheses describe the relative populations of detectable conformers by NMR (yellow squares denote the conformationally homogeneous peptides (>98%), and the gray ones denote the peptides showing a preferred conformation (>80%) on the NMR time scale).

Template Structures. Out of the 16 peptides selected, 15 are grouped into five different classes by virtue of the site of their cis peptide bond (**10** could not be characterized due to spectral overlap). It should be noted that cyclic pentapeptides still have considerable conformational flexibility.[34] Flipping of the plane of the peptide bond by synchronous rotation of adjacent Φ and Ψ angles is often observed and can be fast on the NMR time scale. Hence, the conformations shown in the figures are preferred structures.

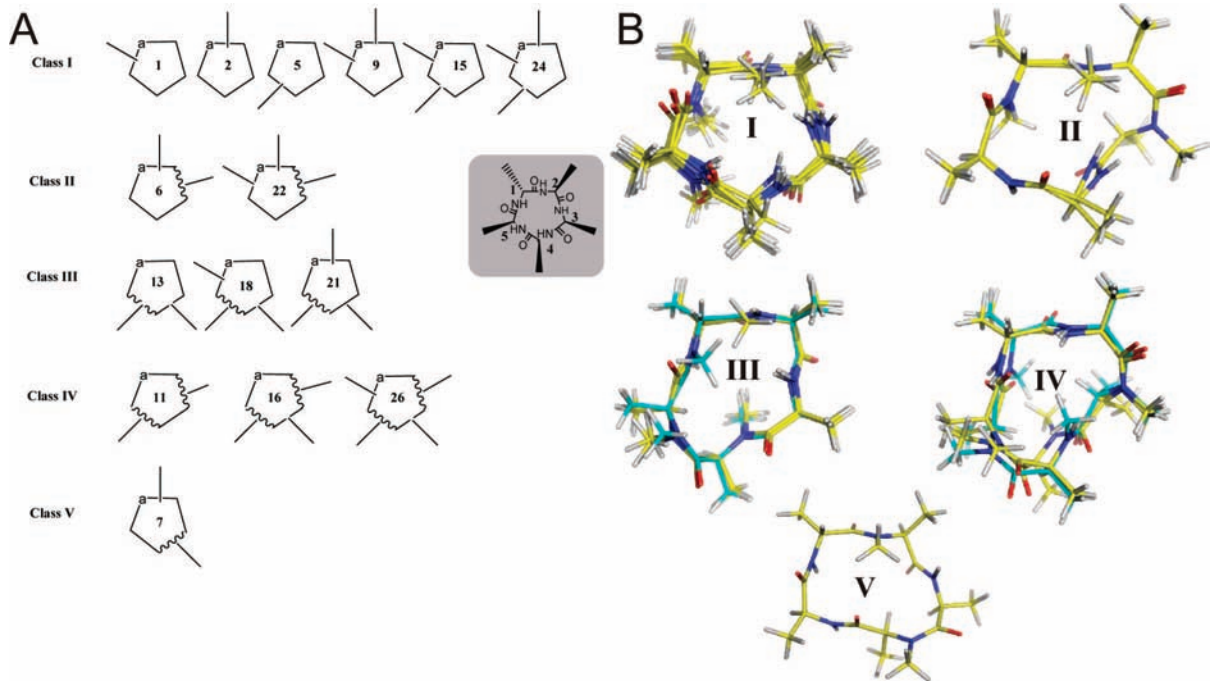


FIGURE 5. (A) Classes of N-methylated cyclo(-D-Ala-L-Ala$_4$-) (wavy lines indicate cis peptide bond; a indicates D-Ala^1) and (B) superimposed backbone conformation of the members in each class. Peptides **18** and **26** are in cyan, highlighting differences in peptide bond orientation caused by the N-methyl group.

Class I consists of six peptides with all trans peptide bonds, having N-methylation at D-Ala^1, Ala^2, and Ala^5 or a combination of these three sites (Figure 5). *Class II* contains peptides having a cis peptide bond between Ala^2 and Ala^3. *Class III* contains the three peptides with a cis peptide bond between Ala^4 and Ala^5. One notable difference in this class is the orientation of the D-Ala^1 N-methyl group in **18**, which undergoes a flip of about 180° from its preferred orientation (projecting above the plane of the ring in Figure 5) as a consequence of strong steric clash between the N-methyl group and Ala^5 methyl group. *Class IV* peptides show the characteristics of both classes II and III, exhibiting two cis peptide bonds between Ala^2 and Ala^3 and Ala^4 and Ala^5. *Class V* contains a unique peptide with a cis peptide bond between Ala^3 and Ala^4. All the other peptides with an Ala^3−Ala^4 N-methylated peptide bond, that is, **13**, **16**, **18**, **21**, and **26**, exhibit a trans peptide bond. This pattern probably arises from the fact that the parent peptide (**4**, Figure 4) with the N-methylated Ala^3−Ala^4 peptide bond exists in a 1:1 equilibrium between cis and trans conformers by NMR. Thus, N-methylation at Ala^2 shifts the equilibrium strongly toward a cis orientation whereas N-methylation at any other site shifts toward trans.

The Ala^5−Ala^3 region of the cyclic pentapeptide is conserved, and N-methylation does not introduce cis peptide bonds. Of the conformationally homogeneous peptides (>98%), six out of seven have N-methylated D-Ala^1 and show a Φ angle close to 120°.[19] A similar N-methyl scan of a cyclic hexapeptide cyclo(-D-Ala-L-Ala$_5$-) revealed that the N-methylation of D-alanine results in conformational homogeneity, as has been recently confirmed in designing N-methylated cyclopeptidic scaffolds against colon cancer.[35] Thus, the turn-inducing property of N-methylated D-alanine or any N-methylated D-amino acid (except glycine) is at least equal to that of D-proline. This will open a new dimension for the design of β-hairpin conformations[36] in cyclic protein epitope mimetics by using NMe-D-Xaa-L-Pro, NMe-D-Xaa-L-MeXaa, and NMe-D-Xaa-L-Xaa as templates to induce a βII′-turn instead of the conventionally used D-Pro-L-Pro[36] (Figure 6). The clear advantage of this method is the ability to incorporate different N-methylated amino acids, providing much greater flexibility in functionalization of the turn-inducing region.

Systematic Modulation. A clear picture of the conformational modulation by successive N-methylation can be obtained by classifying these peptides based on their N-methylation site (Figure 7). Starting with **5**, N-methylation on either side of the N-methylated peptide bond results in **15** and **13**. N-methylation of D-Ala^1 is tolerated in **15** without introducing any cis peptide bond, whereas N-methylation of Ala^4 introduces a cis peptide bond in **13**, and this pattern is followed upon further N-methylation.

Thus, conformational modulation by N-methylation on cyclic peptide backbone is defined and not irregular. These

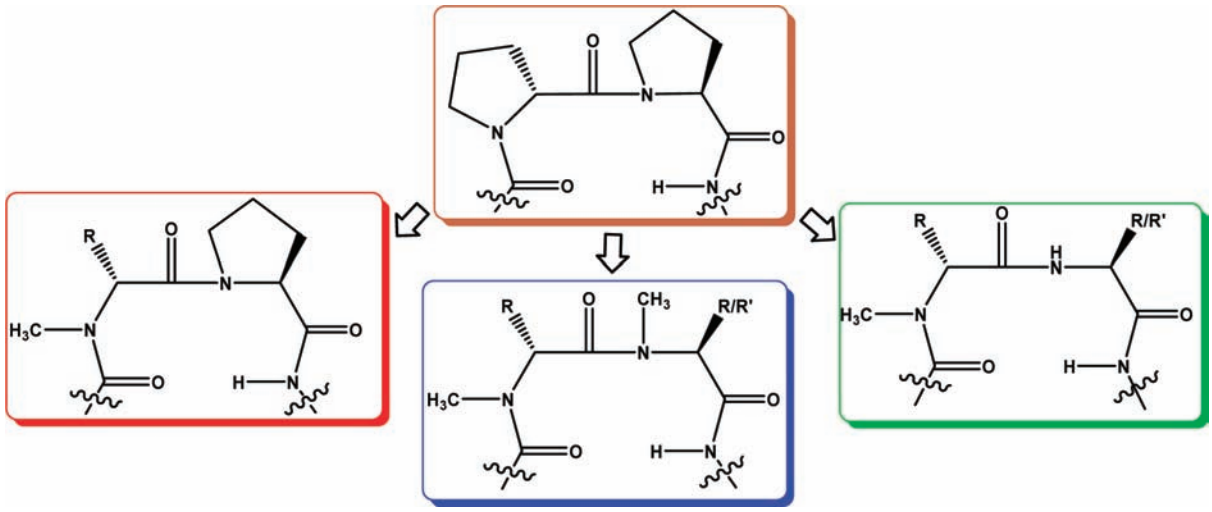


FIGURE 6. Conventional β-hairpin turn inducer, D-Pro-L-Pro, which could be replaced by NMe-D-Xaa-L-Pro, NMe-D-Xaa-L-MeXaa, and NMe-D-Xaa-L-Xaa. R and R′ represent amino acid side chains.

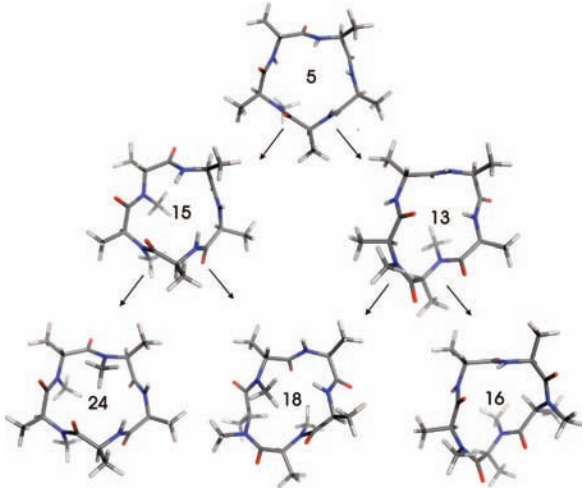


FIGURE 7. Modulation of conformation by successive N-methylation.

described templates can be now used as scaffolds in drug design by substituting alanine with other amino acids representing the pharmacophores of interest. The knowledge of the impact of N-methylation allows the design of N-methylated biologically active peptides without distorting the (bioactive) conformation, leading to modification of pharmacokinetic parameters without loss of biological activity.

Multiple N-Methylation Imparts Oral Bioavailability to Somatostatin Analogs

To investigate possible improvements in the pharmacokinetic properties of bioactive peptides by N-methylation, we chose the well-studied somatostatin system. Somatostatin is a major endocrine hormone and physiological inhibitor of pancreatic and gastrointestinal secretion, of growth hormone, glucagons, and insulin.[37] However, somatostatin has a very short plasma half-life, <3 min, and therefore, there was a need for the development of metabolically stable analogues. In the approach taken by Sandoz, higher metabolic stability of somatostatin analogs was achieved by the following modifications, resulting in the compound dubbed "Sandostatin" (Figure 8):[38] (i) reduction in the size from 14 amino acids to 8; (ii) exchange of Trp^8 with D-Trp^8; (iii) shift of the disulfide bridge closer to the "active loop" (amino acids 6−11); (iv) change of the N-terminal phenylalanine into D-phenylalanine and the C-terminal threonine into reduced threoninol to avoid enzymatic cleavage (Figure 8). This resulted in a dramatically longer half-life *in vivo*. However, Sandostatin (octreotide) is not orally available and must be administered by i.v. injection. Out of a plethora of somatostatin receptor agonists, the earliest was cyclo(-PFwKTF-), discovered rationally by the group of Ralph Hirschmann at Merck Inc. and known as the Veber−Hirschmann peptide (Figure 8). This peptide was reported to be selective toward the somatostatin receptor subtypes sst2 and sst5 and showed excellent activity toward the inhibition of insulin, glucagon, and growth hormone secretion, surpassing the activity of somatostatin itself.[39] However, the compound was administered by subcutaneous injection. Although end-to-end cyclization improved metabolic stability of the peptide in serum, to confer oral bioavailability, the improvement of intestinal permeability and stability against gut enzymes and enhanced uptake from the gut to systemic circulation is necessary. We envisioned that multiple N-methylation of cyclo(-PFwKTF-) might convey improved pharmacokinetic properties, making it orally bioavailable.

Library Approach. A library of all possible N-methylated analogs of cyclo(-PFwKTF-) was synthesized (except the penta-N-methylated analog), resulting in 30 analogs. Out of these

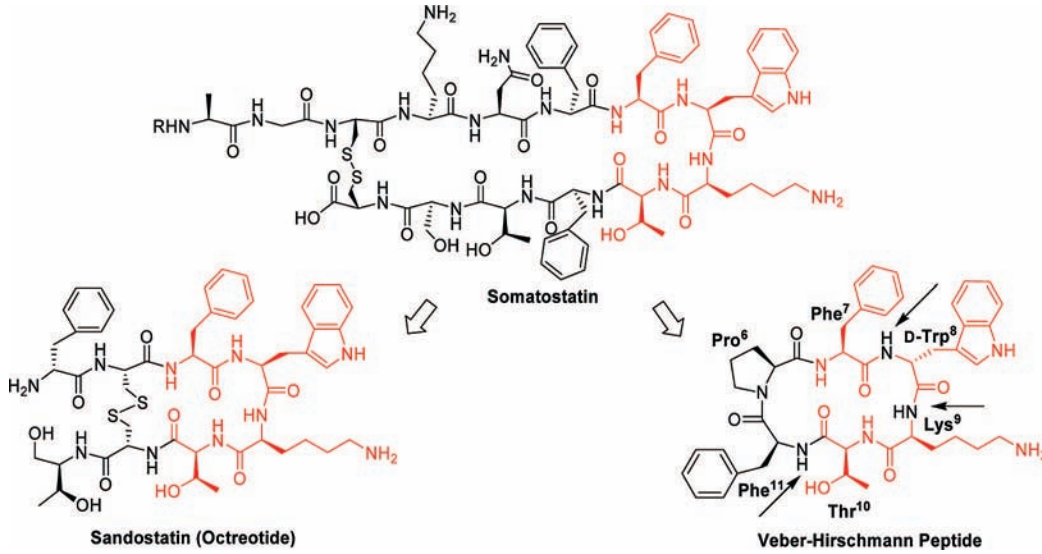


FIGURE 8. Truncation of somatostatin into Sandostatin and the Veber−Hirschman peptide. The active analogs resulted from the N-methylation of the amides shown by arrows.

30, seven analogs showed binding affinity in the nanomolar range toward sst2 and sst5 receptor subtypes (Table 1). Preliminary tests of **S1**−**S8** by oral administration into rats showed only **S1** and **S8** to be significantly taken up into the blood.

TABLE 1. pK_d Values of **S1**−**S8** toward hsst2 and hsst5 Receptors[a]

peptide	N-methylated amino acid	hsst 2 (pK_d)	hsst 5(pK_d)
octreotide	none	9.18	7.71
S1	none	8.01	7.82
S2	Lys^9	8.60	8.19
S3	Phe^{11}	7.93	8.28
S4	D-Trp^8	7.61	7.87
S5	Lys^9, Phe^{11}	7.96	7.39
S6	D-Trp^8, Lys^9	7.60	7.19
S7	D-Trp^8, Phe^{11}	7.16	7.47
S8	D-Trp^8, Lys^9, Phe^{11}	7.21	7.22

[a] Higher pK_d corresponds to higher affinity.

Hence, detailed pharmacokinetic experiments were performed. These eight peptides showed a stable profile in the rat serum; however, a significant difference was found between **S1** and **S8** in their stability against gut enzymes, revealing the stability effect conferred by multiple N-methylation.

The transport mode of these peptides through the intestine revealed interesting facts. We expected multiple N-methylation to confer sufficient lipophilicity to enable the peptides to cross the membrane via the transcellular mechanism (where the peptides interact with the lipophilic membrane of the enterocytes).[40] However, no such trend was observed (Figure 9). All the N-methylated peptides permeate the membrane by a paracellular mechanism through the tight junctions (aqueous extracellular route across the epithelia) with low permeability. Surprisingly a significant increase in the permeability of **S8** was observed, which exceeded even the permeability of mannitol (paracellular marker).

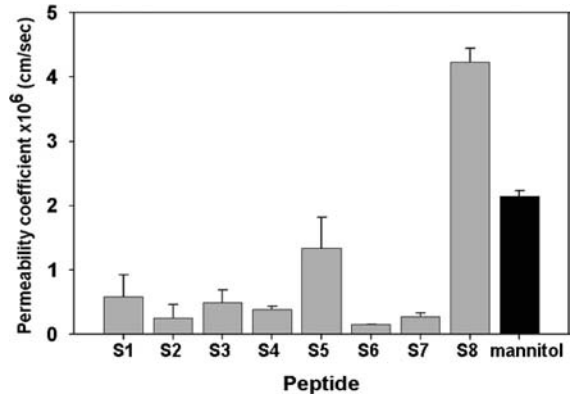


FIGURE 9. Peptide permeability across Caco-2 monolayer.

Conformational Details. Whereas a βII′, βVI conformation was established in one early NMR structure,[45] the presence of a "flat" or "bent" conformation has been an issue of debate regarding the real bioactive conformation of cyclo(-PFwKTF-);[41] Goodman et al. suggested that the peptide exhibits a "bent" conformation,[42] with a kink in the backbone about Phe^7 and Thr^{10}, stabilized by the two additional hydrogen bonds between Pro^6CO−D-Trp^8H^N and Lys^9CO−$Phe^{11}H^N$, forming two closed γ-turns. On the other hand, Veber et al. suggested the "flat" conformation without the two γ-turns to be the bioactive conformation.[43] Interesting evidence was found while screening the interactions of these eight analogs with the liposomal model of the cell membrane (Figure 10), where there was a sudden increase in the liposomal interaction of **S2** (the most active analog) despite being mono-N-methylated.

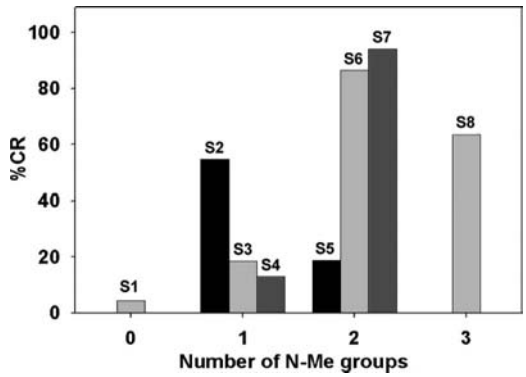


FIGURE 10. The effect of N-methyl position on interaction with the liposomal model of the cell membrane (higher %CR shows higher interaction).

This is due to the enhanced lipophilicity of **S2** compared with **S3** and **S4**, where a "bent" conformation of **S2** is observed in solution compared with the comparatively "flat" conformations of **S3** and **S4** (Figure 11). In the "bent" conformation, the externally oriented amide hydrogens of D-Trp[8] and Phe[11] are involved in a γ-turn with Pro[7] and Lys[9] carbonyls, resulting in the solvent shielding of all amide HNs, making the molecule more lipophilic. This "bent" conformation is observed in cyclo(-PFwKTF-) and **S2** with N-methylated lysine; N-methylation of any other site results in partial to complete loss of this "bent" conformation. Thus, the highest pK_d of **S2** and the gradual decrease in activity with increasing N-methylations suggests that the "bent" conformation is more active than the "flat" one, and a successive loss in activity is seen in moving from "bent" to the "flat" conformer.

A 5-fold difference in the elimination half-life between **S1** and **S8** (15.5 $\pm$ 2 and 74 $\pm$ 6 min, respectively) suggested reduced proteolytic digestion or low hepatic or renal clearance, which is preferable for a good drug. A 10-fold difference in the volume of distribution at steady state for **S1** and **S8** (0.3 $\pm$ 0.1 and 3.7 $\pm$ 1.3 L/Kg, respectively) revealed that distribution of **S8** is not only limited to interstitial fluid and blood but also to biological membranes. The absolute oral bioavailability of **S8** was found to be 9.9%, which is remarkable for a peptidic drug obtained by the relatively simple modification of N-methylation.

Multiple N-Methylation Imparts Subtype Selectivity to Integrin Ligands:

Design Approach. Instead of the commonly used library approach, we envisioned an approach using designed multiple N-methylation, where only the externally oriented amide bonds were targeted.[17] This was based on our experience of the somatostatin analogs, where N-methylation of only the externally oriented amide bonds results in the bioactive analogs, whereas targeting the internally oriented ones distorts the bioactive conformation. To test our hypothesis, we chose to N-methylate a cyclic hexapeptide αIIbβ3 integrin receptor antagonist, cyclo(-G^{1}R^{2}G^{3}D^{4}f^{5}L^6-)[44] (Figure 12, **R1**) in an attempt to confer oral bioavailability. αIIbβ3 is the most abundant integrin on the surface of platelets and mediates formation of thrombi by platelet aggregation. In the final step of blood clot formation, the activated αIIbβ3 binds to the blood glycoprotein fibrinogen to cross-link platelets in a growing thrombus. Thus compounds that compete with fibrinogen in binding to αIIbβ3 can act as potent antithrombotic agents.[45] The preliminary design criterion in this approach is a prior knowledge of the *conformation of the lead peptide*. An added advantage would be knowledge of its active sequence.

Selectivity. The activity and selectivity of the lead compound **R1** and seven N-methylated analogs (**R2**−**R7**) are shown in Table 2. The lead structure was unselective; however, satisfactory activity and selectivity toward αIIbβ3 was first obtained in **R4**, where the Arg[2] was N-methylated. The preferred αIIbβ3 selectivity is due to the hexapeptidic ligands, where the -RGD- recognition sequence is part of the βII-turn (Figure 12B), and particularly by an extended conformation in this β-turn, such that the guanidine and the aspartic acid groups are farthest apart (length of binding pocket: αIIβ3 > α5β1 > αvβ3).[45]

Absence of selectivity in **R1** is due to the conformation of cyclic hexapeptides of the family cyclo(-D-Xaa-L-Xaa$_5$-) (Xaa = all amino acids except proline and glycine) exhibiting a "preferred" βII′ and flexible βII/βI-turn. This flexibility about the recognition motif RGD results in lower selectivity. However, when

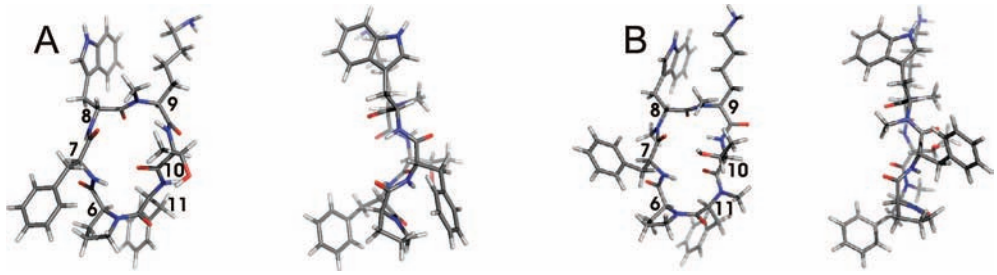


FIGURE 11. Front and side view of the solution conformations of (A) **S2** (note the "bend" in the backbone) and (B) **S8** (complete loss in the "bend" by N-methylation).

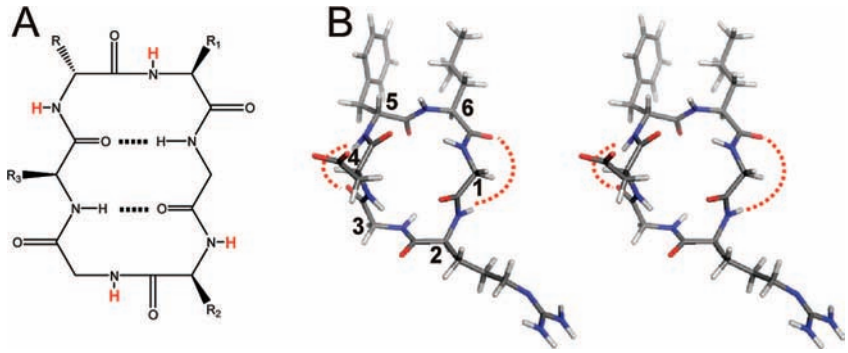


FIGURE 12. (A) **R1** with two β-turns (solvent exposed amides (red) targeted for N-methylation) and (B) stereoview of cyclo(-GRGDfL-) (**R1**). Note the stabilizing γ-turns about Asp^4 and Gly^1 (similar as that discussed above for the somatostatin hexapeptides).

TABLE 2. IC_{50} (nM) of the N-Methylated Analogs and Cyclo(-GRGDfL-) toward Different Integrins[a]

no.	analogue	$\alpha5\beta1$	$\alpha v\beta3$	$\alpha IIb\beta3$	$\alpha v\beta3/\alpha IIb\beta3$
R1	c(-GRGDfL-)	740	100	195	0.5
R2	c(-GRGD**f**L-)	3900	103	560	0.2
R3	c(-GRGD**f**L-)	4300	490	2000	0.2
R4	c(-G**R**GDfL-)	1200	770	12	64
R5	c(-GRGD**fL**-)	>20000	1200	620	2
R6	c(-G**R**GD**fL**-)	~20000	1300	15	86
R7	c(-G**R**GDfL-)	>20000	2730	165	16
R8	c(-G**R**GD**fL**-)	>20000	12,200	30	406

[a] N-methylated residues are in bold.

Arg^2 is N-methylated, the flexibility is reduced and the βII/βI-turn is presented in an extended orientation, affecting both the activity and selectivity of the ligand.

IC_{50} values suggest that N-methylation at D-Phe^5 is not tolerated; whereas N-methylation at Leu^6 is favored and results in enhanced selectivity of **R6**. The most surprising result was the activity and selectivity profile of **R8**, where all the three sites are N-methylated, in contrast to **R5**, where N-methylation of D-Phe^5 results in the loss of activity. Thus, one would also expect a further loss in the activity in **R8**; instead a tremendous enhancement in the selectivity was observed with a slight loss in activity.

The solution conformations reveal the reduced flexibility in the β-turn about Arg^2 and Gly^3 in **R4**; in addition, we observe a kink in the backbone about Gly^1 and Asp^4 in **R1** (Figure 12B), a pattern that was also observed in the somatostatin analogs and is probably typical of the cyclo(-D-Xaa-L-Xaa$_5$-) class of peptides. This kink helps in the formation of a γ-turn about Gly^3, preventing an extended conformation in the β-turn and bringing the side chains of Arg^2 and Asp^4 in close proximity, which is favored for binding to $\alpha v\beta3$. This kink is lost partially by the N-methylation of Arg^2 resulting in the loss in binding to $\alpha v\beta3$ in **R4** (Figure 13A). A total loss in the kink is observed by N-methylation of D-Phe^5 in **R7** and in **R8** (Figure 13B), presenting the peptide in a "flat" conformation.

Docking (Figure 13C) revealed differences in the upper part of the peptides, where N-methylation of D-Phe^5 in **R8** compared with **R4** lowered the $\pi-\pi$ interaction between the phenyl rings with β3-Tyr^{122} and a change in the preferred orientation of Leu^6 carbonyl group to form hydrogen bond with β3-Arg^{214} side chain. Unfortunately, docking could not give a clear distinction between the binding of the ligands to $\alpha v\beta3$ and $\alpha IIb\beta3$.

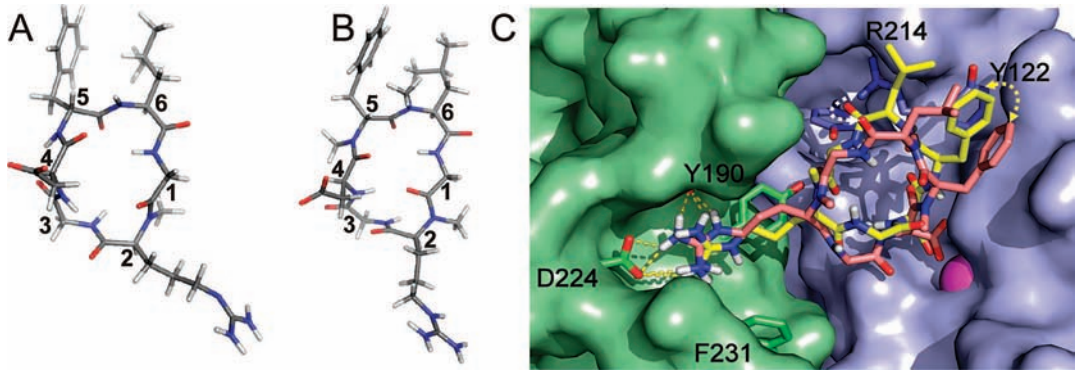


FIGURE 13. Conformation of (A) **R4** and (B) **R8** and (C) docked **R4** (yellow) and **R8** (pink) in the $\alpha IIb\beta3$ integrin. αIIb subunit is represented by the green surface, $\beta3$ by the violet, and metal ion by the magenta sphere. Reproduced with permission from ref 17. Copyright 2007 American Chemical Society.

We have shown that a designed approach to multiple N-methylation based on the conformation of the stem peptide leads to the development of very potent and receptor subtype selective ligands. Unfortunately, these peptides showed low permeability in the Caco-2 test. Although these peptides might show enhanced duration of action due to proteolytic stability, we discontinued the project due to lack of enhanced permeability.

Summary

Mono- and multiple N-methylations of cyclic peptides were investigated to elucidate their remarkable conformational modulation ability by imparting steric constraints in the peptidic backbone and to improve the pharmacokinetic profile of the peptides in order to be used as drug leads. The development of simplified and easily scalable synthesis of N-methylated amino acids in solution and of peptides in solid phase accelerated the N-methyl scanning technique of peptides. The elucidation of the conformational impact of N-methylation on cyclic peptides highly facilitates the design of bioactive peptides by "spatial screening", wherein the side chains in the template structures are functionalized by appropriate pharmacophores. The improvement of oral bioavailability by multiple N-methylation is a significant advance toward the development of peptide-based therapeutics, which has hampered over the years due to poor pharmacokinetic properties. Multiple N-methylation resulted in enhancement in the activity and selectivity of receptor subtypes using either library or designed approaches and helps in understanding finer details of the bioactive conformation. Thus, with these diverse properties, we foresee a bright future for peptide chemistry by multiple N-methylation toward their development as a therapeutic prototypes.

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FOOTNOTES

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