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Improving oral bioavailability of cyclic peptides by *N*-methylation

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ABSTRACT

The renaissance of peptides in pharmaceutical industry results from their importance in many biological functions. However, low metabolic stability and the lack of oral availability of most peptides is a certain limitation. Whereas metabolic instability may be often overcome by development of small cyclic peptides containing D-amino acids, the very low oral availability of most peptides is a serious limitation for some medicinal applications. The situation is complicated because a twofold optimization – biological activity and oral availability – is required to overcome this problem. Moreover, most simple "rules" for achieving oral availability are not general and are applicable only to limited cases. Many structural modifications for increasing biological activities and metabolic stabilities of cyclic peptides have been described, of which *N*-alkylation is probably the most common. This mini-review focuses on the effects of *N*-methylation of cyclic peptides in strategies to optimize bioavailabilities.

1. Introduction

1.1. General remarks

Peptides are among the most important biomolecules. For instance, they function as hormones,[1] neurotransmitters[2] or as signaling molecules in the immune response.[3] In higher organisms, peptides are usually expressed locally, tend to be flexible,[4] and their short half-lives under physiological conditions facilitate rapid removal after they have fulfilled their functions.[5] In addition, natural peptides are found as secondary metabolites in bacteria, plants and marine organisms.[6]

In medicinal chemistry, peptides are often used as protein-binding site mimetics to interfere protein-protein interactions.[7] Requirements for peptidic drugs are different to most physiological peptides insofar as they should have higher physiological stabilities. For many applications, peptides should ideally be orally available, but most do not conform to the guidelines outlined by Lipinski[8,9] or Veber.[10]

Short half-lives of peptides *in vivo* are a consequence of rapid degradation by *exo*- and *endo*-peptidases in blood serum.[11] These enzymes favor peptidic substrates that have extended and flexible conformations. Degradation by *exo*-peptidases can be suppressed e.g. by incorporation of *N*-terminal D-amino acids, *C*-terminal reduction of the carboxylic acid into the corresponding alcohol[12] or head-to-tail cyclization.[13,14] The latter has the advantage of bending the peptide chain and rigidifying the conformation and in consequence also hinders cleavage by *endo*-peptidases. Hence, cyclization is the easiest way to extend a peptide's half-life *in vivo*.[15] However, cyclization can lead to a loss of biological activity if the reduced flexibility fixes an inactive conformation, but there are many examples where cyclization has been shown to increase biological activities.[13,16,17] For a comprehensive overview of cyclization methods, we refer to book chapters.[18–20] Simultaneously, incorporation of D-amino acids[13,16–18,21–23] and/or *N*-methylation of amide bonds can increase metabolic stability by conformational control or steric hindrance.[24–26]

To obtain both stable and bioactive as well as orally bioavailable peptidic drugs a twofold optimization is necessary and leads to two possible approaches in practice:

i) Structural optimization for biological activity, followed by the conversion of the lead peptide into an orally bioavailable derivative, retaining its biological activity.
ii) Development of orally available peptide backbones and utilizing them as scaffolds for the subsequent introduction of functionality in a stereocontrolled manner to achieve biological activity.

An example of the first approach was the successful conversion of the somatostatin-analog, the *Veber-Hirschmann*-peptide,[27] into

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an orally available cyclopeptide *cyclo*(-Pro-Phe-*N*Me-D-Trp-*N*MeLys-Thr-*N*MePhe-) (=*c*(-PF*w*KT*F-)) (**2**) by threefold *N*-methylation (amino acids in D-configuration presented in small letters, the *N*-methylated peptide bond is indicated by *)[28] resulting in a derivative having high oral bioavailability (**F**).[29]

The second strategy – introduction of functionality in an orally available backbone to our knowledge has not yet successfully realized. Recently, a number of cyclic hexapeptides exhibiting a good permeability[30–36] have been found, but so far **no bioactive peptide retaining its oral availability** has been developed following this approach.

Nature has created biologically active peptides that can be orally bioavailable. The hepta-*N*-methyl undecapeptide cyclosporin A[37] (**1**) is an orally available immunosuppression drug.

This review on bioavailable *N*-methylated cyclic peptides is more narrowly focused than prior reviews on bioavailable peptides.[39–43] It emphasizes structural issues hence excludes discussions of pharmaceutical formulation to enhance bioavailability,[44] or the use of cell-penetrating peptides[45–47] and peptides crossing the blood-brain-barrier[48,49] are reviewed elsewhere.

1.2. Mechanisms of intestinal drug uptake

There are several pathways for drugs to traverse from the apical to the basolateral side of the epithelial cells in the brush border membrane of the gut. In the paracellular pathway, the drug passes between the cells through the tight junctions in a passive manner (Fig. 2, **a**).[50,51] Both the size and rigidity of the compound play a

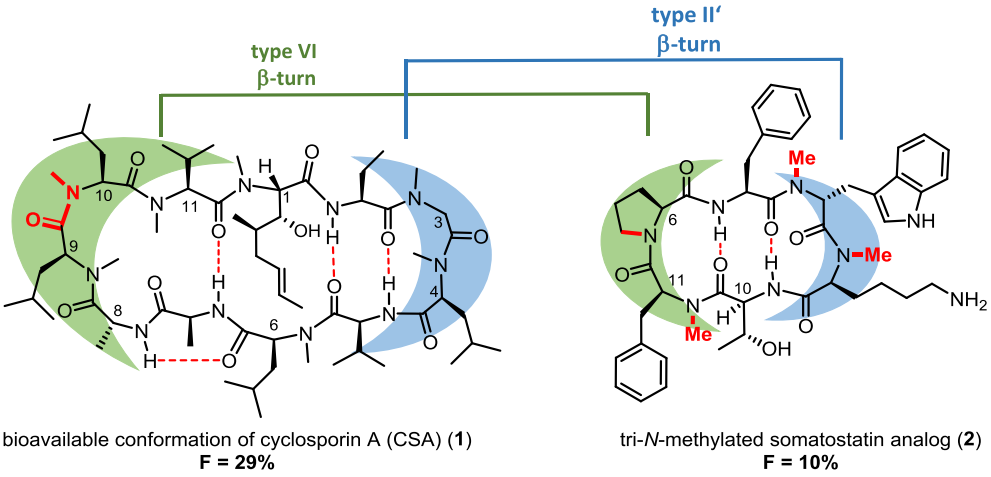


Fig. 1. Comparison of two permeable peptides: on the left the schematic model of the bioavailable conformation of cyclosporin A (CSA) (**1**) exhibits a βVI-turn (highlighted in green) including a *cis*-peptide bond between MeLeu9 and MeLeu10 (highlighted in red) and a βII'-turn about Sar3-MeLeu4 (highlighted in blue); it has a bioavailability of **F = 29%** (where **F** is the ratio of orally administered compound to intravenously administered compound measured in the blood).[38] On the right the tri-*N*-methylated somatostatin analog (**2**) has all externally orientated NH groups methylated, and the related structure motifs are highlighted; it has an oral bioavailability of **F = 10%** in rats.[29]

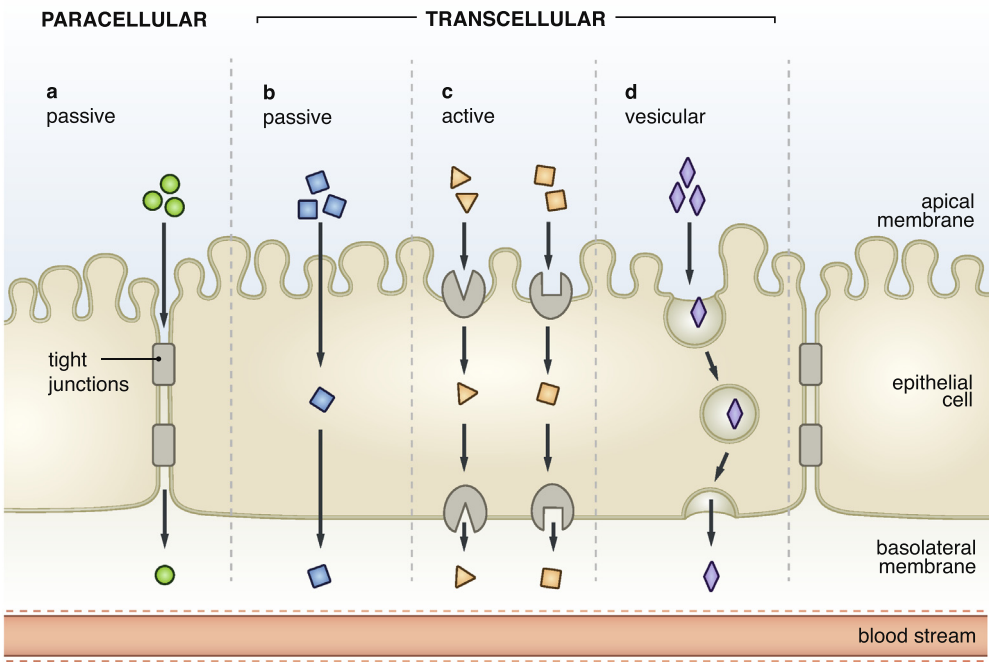


Fig. 2. Intestinal uptake requires compounds to pass the brush border membrane of the gut from the apical to the basolateral side of the epithelial cells. The two main pathways are paracellular, passive (**a**) between the cells and through the tight junctions and transcellular, through the cells. For the transcellular pathway different mechanisms are known. Passive (**b**) diffusion through the cell membrane, carrier-mediated active (**c**) transport and vesicular (**d**) uptake.

role in this process. Pharmaceutical formulations can influence mesh size that determines the paracellular uptake.[14,44,52] In the transcellular pathway, drugs cross the epithelial cells *via* passive, active or vesicular routes (Fig. 2, **b–d**).[53] Passive transport is controlled by the peptide's lipophilicity and rigidity.[52] Active transport is defined by various types of membrane proteins,[54] which conduct the peptide internalization, and for which turnover rates of the membrane transport proteins are critical. Overall, several of these mechanisms act synchronously and the contributions to the permeability of a compound depend on the structure and properties of the drug. Hence, it is not surprising that no simple rules have been found to increase oral availability.

1.3. Synthesis of N-methylated peptides

Some protected *N*-methylated amino acids are commercially available and can be directly used in conventional peptide synthesis, but many have to be chemically synthesized. Briefly, three prevalent methods for syntheses of *N*-methylated peptides have emerged. *N*-Methylated amino acids without functional groups are accessible *via* the *Freidinger* method[55] (Scheme 1, **A.I.**). Functionalized amino acids can be *N*-methylated as *o*-NBS-protected amino acids (Scheme 1, **A.II.**),[56] this protecting group is also applicable to *N*-methylation of an *N*-terminal amino acids in solid phase peptide syntheses under *Mitsunobu*-conditions.[57–61] The advantage here is that the *o*-NBS-group is only cleaved-off after *N*-alkylation, but remains stable under cleavage conditions without a previous *N*-alkylation (self-cleaning procedure, Scheme 1, **B.**).[60]

Recently White et al. published a method to methylate the solvent exposed NH-groups in cyclic peptides on solid support in which the anchored peptide is treated with lithium-*tert*-butanolate and methylated with methyliodide in DMSO (Scheme 1 **C.**).[31] This strategy provides a direct way to explore the influence of externally orientated methylated amide bonds on passive transport in bioavailability research.

Another synthetic method towards *N*-methylated peptides provides the flexizyme mediated synthesis of aminoacetylated *t*-RNAs with unnatural amino acids by the *Suga's* group.[62]

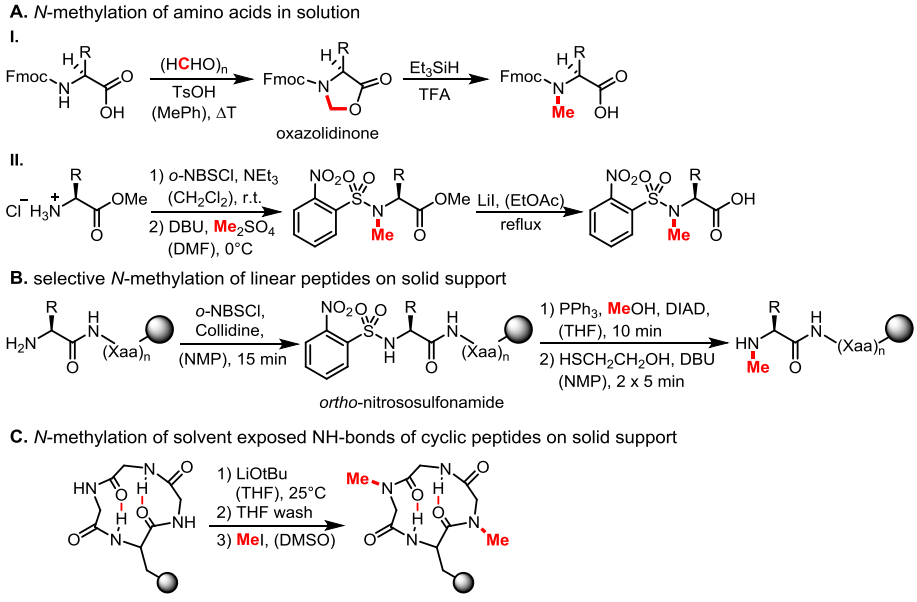
2. Using *N*-methylation for improving bioavailability

2.1. General remarks to N-methylation

It is difficult to attribute the effects of any particular chemical modification, such as an *N*-methylation, on bioavailability to a single effect. Any change in structure also has a strong influence on all physical, chemical and biological properties of the peptide.[19,26,63] *N*-Methylation, similar to proline,[64] increases the steric hindrance, and this results in an increased population of the *cis*-conformation of the amide bond,[65–67] which is easily detectable by NMR spectroscopy.[65] Even in smaller cyclic peptides non-*N*-methylated amide bonds usually are, though not always,[67] *trans* configured (Z-conformation). Of course, *N*-methylation of amides in cyclic peptides removes H-bond donor capabilities, but, in our opinion, the importance of this effect for controlling the backbone conformation of cyclic peptides is often overestimated.[63] In our experience, chirality of the amino acids and their positions in the sequence appears to have more impact on conformations of cyclic penta- and hexa-peptides than stabilization by intramolecular *H*-bonds does.[63]

We[29,32] and others[31,36] speculated that *N*-methylation of all solvent exposed NH-groups could be a key to achieve oral availability. However, as we will see below, this speculation does not hold.[68] Simple parameters such as lipophilicity, the lack of externally oriented NH groups and the number of *N*-methylations seem to be important, but cannot generally explain permeability. Each factor may contribute to permeability, but distinct conformational features seem to be more important.[33,69]

In addition to *N*-methylation, a range of structural properties and chemical modifications influence the bioavailability of peptides. Pioneering studies by Borchardt et al. identified the influence of size,[70] cyclization,[71] β-turns[72] and restricted conformations[71] on the passive diffusion across the Caco-2 cell[73–76] monolayers. The influence of D-amino acids[18] and α-methylation[77] were also examined with respect to their influence on bioavailability. Furthermore, different substitutions or blocking of the hydrogen bond donor of the amide bond were examined for their influence on lipophilicity of cyclic peptides. Depsipeptides,[40] cyclo-alanines,[78]



Scheme 1. Methods for *N*-methylation of amino acids and peptides: For unfunctionalized amino acids, the Freidinger method is preferred (**A.I.**). For functionalized amino acids the use of o-NBS protecting group is established as well in solution (**A.II.**) as on solid support (**B.**) under Mitsunobu-conditions. *N*-methylation of solvent exposed NH groups of cyclized peptides on solid support (**C.**). (o-NBS is *ortho*-nitrobenzenesulfonyl).

aziridines,[79] exocyclic amide bonds[80] and sulfur or oxygen containing heterocycles[35,81] remove not only the NH-bond, but also affect the rigidity and the shape of the cyclic peptides.

2.2. Improving bioavailability: lessons from nature – Cyclosporin A

Naturally occurring, biologically active, permeable peptides mostly belong to the class of *N*-methylated or peptoidic cyclic hexapeptides, and to larger rings. Cyclosporin A (**1**), an orally bioavailable peptidic drug is a fungal metabolite discovered by Sandoz in the 1970's for its effect as an immunosuppressive drug.[37,82,83] It is a hepta-*N*-methylated undecapeptide,[37] which we structurally elucidated more than 30 years ago for its conformation in lipophilic solvents and in the crystallized form.[84,85] It later turned out that the conformation in polar solvent and in water[86] or when bound to its receptor cyclophilin[87–95] is different, involving a conversion of a *cis*-peptide bond (marked in compound **1** in Fig. 1) between Me-Leu9 and Me-Leu10 into the *trans*-conformation.[87–95] Cyclosporin A violates all of the Lipinski's rules on oral bioavailability,[8,9] but is orally available (**F = 29%**).[38] In case of CSA the uptake is strongly dependent on the patient and many other factors like pharmaceutical formulation, hence, a range for the different bioavailabilities is mentioned in literature **F = 15–50%**,[34] see also citations there. It is evident that *N*-methylation, intramolecular hydrogen bonds, lack of externally oriented NH groups, lipophilic side chains (especially many leucines) and structural motifs,[33] (specifically one βII' and one βVI-turn containing a *cis*-peptide bond) may help to increase oral availability.

2.3. N-Methylation of externally orientated NH-groups to enhance passive diffusion

N-Methylation might be used for blocking externally orientated amide bonds to improve the passive diffusion of cyclic peptides. A routine method to identify these NH-protons in H-bond acceptor solvents like DMSO is to use temperature gradients ($\Delta\delta_{NH}/\Delta T$) in ^{1}H NMR spectra.[13] Solvation of NH-protons shift these resonances downfield, while entropic effects increasingly disrupt these H-bonds as the temperature is raised. *N*-Methylation of externally oriented NH groups tend to increase the permeability of a peptide,[36] but this is only one parameter; there are examples which contradict this simple rule.[68]

2.3.1. Veber-Hirschmann peptide

Multiple *N*-methylations of the NH-groups in the *Veber-Hirschmann*-peptide[27] has been investigated (30 derivatives, the penta-*N*-methylated analog could not be prepared). Eight of these derivatives were active for some members of the somatostatin receptors.[29] Only one of them (compound **2** in Fig. 1) exhibited permeability across the Caco-2 cell membrane (68% increase compared to the non-*N*-methylated) and was orally available in rats (about **F = 10%**), whereas the non-*N*-methylated peptide and the other *N*-methylated peptides were not bioavailable at all.[29] Comparison of the bioavailable derivative[29] and the *Veber-Hirschmann*-stem peptide[96] revealed that the conformation was not altered by *N*-methylation, and all the externally oriented NH-groups were *N*-methylated.

Stabilities of the non- and the tri-*N*-methylated peptide against enzymes of the brush border membrane was also investigated. These membranes contain a variety of peptidases and play a major role in the digestion of peptides and proteins in the gut wall.[22] The tri-*N*-methylated peptide, in contrast to the non-*N*-methylated one, was completely stable under these assay conditions for 1.5 h.[29] Stability against proteases is known also for CSA (**1**).[97]

In addition, a diastereomer of peptide **2** was synthesized (substitution of L-MePhe11 with D-MePhe11). Remarkably, not only the activity was completely lost, but also the membrane permeability was strongly reduced in the Caco-2 cell assay. The substitution resulted in a *trans* peptide bond between D-MePhe11 and Pro6 and hence in a type II' β-turn, instead of a type VI β-turn in peptide **2**.[29] The study of the *Veber-Hirschmann*-peptide clearly confirms the correlation that multiple-*N*-methylation only improves the bioavailability if distinct conformational characteristics are simultaneously satisfied.

2.3.2. Permeable lipophilic peptides (PLPs) of the type cyclo(-Leu$_4$-Pro-Tyr-)

To examine the influence of the externally orientated NH-groups, a library based on the hexapeptide sequence *cyclo*(-Leu$_4$-Pro-Tyr-) with different D-amino acid substitutions was synthesized. These cyclic peptides could be selectively methylated on externally orientated NH-groups for some of the scaffolds (see Scheme 1 **C.**). From 29 possible diastereomers (out of 32 theoretically possible) that cyclized well on solid support, 12 had a relatively high similarity in their constitution converging in a single structure (>95%).[31] Two of those scaffolds (**3** and **4**) were examined in hydrogen-deuterium exchange studies and compared with their non- and per-*N*-methylated compounds in PAMPA (parallel artificial membrane permeability assay).[98,99] Solution NMR and the hydrogen deuterium exchange studies clearly indicate that the non-*N*-methylated NH groups are involved in intramolecular hydrogen bonds. Backbone *N*-methylation and amino acid constitution fix the peptide scaffold, thus stabilize the hydrogen bonds. The resulting structure is schematically shown in Fig. 3.

An alternative structure of peptide **4** would result from a small ψ an ϕ twist around the amide bond between Tyr6 and D-Leu1, and binding the internally orientated NH groups of the Leu3 to the CO of *N*Me-Tyr6 (see Fig. 3). Such small structural changes are difficult to differentiate *via* NOE. The resulting structure forms a classical βII'-turn about *N*Me-D-Leu1 and *N*Me-Leu2. Even if this conformation is not the most populated one in solution, it could be accessed during transport, hence, the β-turn structure in this molecule also might be important (see below).

In PAMPA studies, non- and per-*N*-methylated derivatives of compounds **3** and **4** exhibited significant lower permeability (totally ~10%) compared to the externally methylated **3** (totally ~40%) and **4** (~50%). In cell-based permeability assays (with RRCK (Ralph Russ canine cell line)[100]), the non-*N*-methylated compounds showed very low permeability, whereas their externally methylated counterparts were three- to ten-fold more permeable. Compound **4** had a significant lower microsomal stability in human liver microsomes (HLM), so only **3** was tested *in vivo* to determine the oral bioavailability (**F = 28%** in *Wistar-Han* rats; comparable to cyclosporin A).[31] In conclusion, each scaffold has an optimal number of *N*-methyl groups to improve its passive membrane diffusion. For the best PLP **3** the backbone structure is similar to the tri-*N*-methylated somatostatin analog **2**.[29] Recently, the same research group developed a combinatorial library for the synthesis and the screening of permeable peptides with the same amino acid sequence, but with different stereo information and *N*-methyl pattern.[101]

In a previous study, the same amino acid sequence was examined on passive diffusion without *N*-methylation. In the PAMPA test system, a cyclic peptide was found with a higher permeability than cyclosporin A. In this compound, all NH-groups are involved in intramolecular hydrogen bonds.[102]

Attempts to substitute one Leu amino acid by a more polar side chain illustrated the restricted scope for introducing functional groups in these PLPs. Exchanging Leu3 by Tyr, Trp, Asp or Lys let drop the availability in RRCK cell assays. Only with the substitution of Leu3 by Thr polarity could be introduced in peptide **3** without losing the bioavailability. The methyl group in β-position of Thr

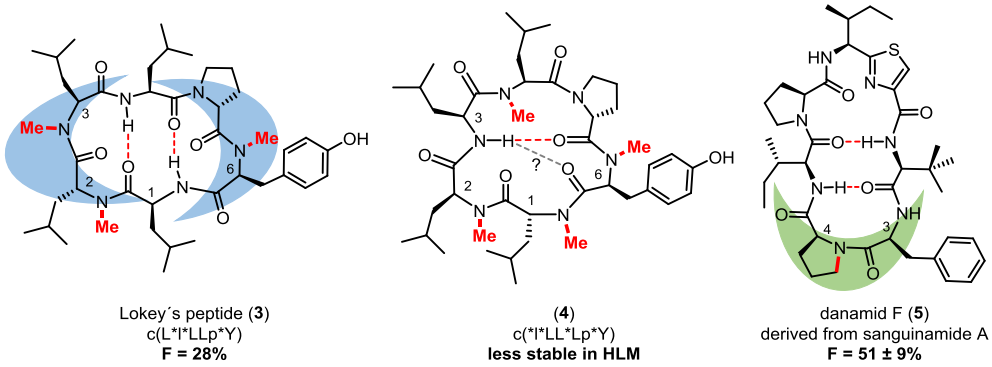


Fig. 3. The permeable lipophilic peptides **3** and **4**. Peptide **4** is less stable to human liver microsomes (HLM). Peptide **3** has the highest permeability (**F = 28%**) and a similar structure to some known bioavailable peptides (highlighted in blue).[31] For a proposed alternative structure of peptide **4** see in the text above. An optimization regarding its bioavailability resulted in the highly bioavailable danamid F (**5; F = 51 ± 9%**).[35]

was found to be essential. In case of an exchange to Ser the availability in RRCK cells and in rats (**F = 2%**) decreases as well. It can be assumed that the hydroxyl group of Thr is internally oriented to form intramolecular hydrogen bonds and the β-methyl group is blocking the polarity of the molecule. For the Leu3Ser mutant peptide an oral bioavailability of **F ~ 24%** was determined in rats.[103] The tolerance of Thr as a "polar amino acid" is also observed for the *Veber-Hirschmann* peptide (**2**) (Fig. 1).[29]

2.3.3. Further examples for lipophilic peptides

The importance of the non-polar aliphatic (especially Leu) side chains for the bioavailability of cyclic penta- and hexaleucines lacking *N*-methyl groups was recently highlighted by Hill et. al.[34] In Caco-2 assays, the bioavailability was comparable to cyclosporin A (**1**) in male *Wistar* rats: *cyclo*[(L-Leu)$_5$], **F = 4.0 ± 1.7%;** *cyclo*[(D-Leu)(L-Leu)$_5$], **F = 8.5 ± 1.1%;** *cyclo*[(L-Leu)$_6$], **F = 17.3 ± 5.7%;** cyclosporin A, **F = 22.5 ± 7.8%**).[34]

In another example, oral bioavailability of the cyclic heptapeptide sanguinamide A was achieved by systematic rigidification and shielding of the externally oriented NH bond of Phe3 by the neighbored sterically demanding *t*Bu-Gly.[35] Here, the *N*-methylation of the solvent exposed NH of Phe3 resulted in a more flexible, but more lipophilic peptide (shown in an NMR study and in RRCK assay); apparently, enhanced flexibility reduced the oral bioavailability. The most permeable heptapeptide **5** in this study, the danamid F (see Fig. 3), showed an oral bioavailability of **F = 51 ± 9%**. Peptide **5** contains a β-turn between Phe3-Pro4 which is an important conformational element in other permeable cyclic hexapeptides (see below).[33] In addition, the upper part of the molecule **5** in the presentation of Fig. 3 can also be considered as a β-turn extended by the heterocyclic ring.

Another study by the *Lokey* group examined the influence of β-branched substitutions of the amino acids and the *N*-methylation of the external oriented, and hydrogen bonded, NH groups of the cyclic heptapeptide sanguinamide A. *N*-Methylation of the solvent exposed NH groups increased permeability in Caco-2 cell assays by a factor of about 6, while the influence of β-branching was less effective (factor of ~1.5).[104] Biological activities of the modified peptides have not been reported so far.[35]

Increasing lipophilicity of cyclic peptides is widely regarded as a way to enhance passive diffusion, but within the limitation that high lipophilicity is a disadvantage for potential drugs (see Lipinsky rules). Most peptidic drugs contain polar functional groups and substitution of Leu residues by polar amino acids tends to reduce permeability.[31,103] A few lipophilic and bioactive cyclic hexapeptides are known, but to the best of our knowledge their bioavailability (especially after modification via *N*-methylation) have not yet been investigated.[105,106]

2.4. The role of N-methylation in bioavailability – beyond passive diffusion

To determine the structural influences on bioavailability beyond passive diffusion, cell-based permeability assays – such as RRCK,[100] MDCK (Madin-Darby Canine Kidney)[107] or Caco-2[76,108] – have to be conducted and analyzed. RRCK cell monolayers provide a good tool to measure passive transport with minimal interference from active transport mechanisms compared to Caco-2 cells,[31] hence the Caco-2 model has been used more widely to examine the effects of active transport on bioavailabilities of cyclic peptides.[34] Caco-2 cell assays tend to provide a reliable prediction of *in vivo* absorption of drugs with lower molecular weight.[73] Transporter proteins and efflux proteins expressed on Caco-2 cells mimic a variety of transcellular transport pathways as well as the paracellular pathway.[75] Differences between the apical-to-basolateral (a-b) and the basolateral-to-apical (b-a) permeability rates (see Fig. 2) indicates the participation of carrier-mediated transport, whereas identical permeabilities in both directions are indicating passive diffusion or active transports identical in both directions. Caco-2 assays are highly dependent from the preparation of the cell layers and *absolute values* strongly differ in different laboratories; consequently, comparison with widely used standards like testosterone are recommended.[109]

2.4.1. Identifying common backbone motifs for permeability: cyclic-hexaalanines (CHAs)

To investigate the role of conformation and flexibility of peptide backbones, a large library of *N*-methylated cyclic hexapeptides (54 out of 64 possible differently *N*-methylated peptides) of the general formula *cyclo*[(D-Ala)(L-Ala)$_5$] was examined by the Caco-2 cell assay. Ala was used to negate the effects of functionalized side-chains to focus on backbone conformations.[32,33]

Surprisingly, the permeability of all these compounds were totally diverse, ranging from no permeability at all to permeation of the Caco-2 cell membrane with rates similar to testosterone (100 % orally available). Out of this library, eight peptides were found to have a high Caco-2 cell permeability, but none permeated the artificial membrane in the PAMPA.[32] Furthermore, seven of these eight peptides featured *N*-methylation adjacent to the D-Ala.[32] An *N*-methylated D-amino acid (also D-proline) is known to be a powerful inducer of a βII'-turn (in the i + 1 position of the turn). Thus the permeability data in this series is in agreement to the identified importance of β-turns in permeable peptides.[33] Beside the fact that these peptides have either two or four *N*-methyl groups, there was no correlation between the permeability and any single parameter, such as the number of *N*-methyl

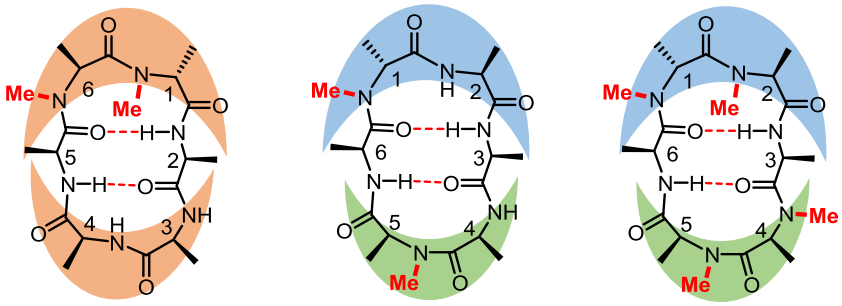


Fig. 4. In a library of cyclohexaalanines (CHAs) the three peptides **6**, **7** and **8** show high permeability in Caco-2 cell assays and they are homogenous in 1H NMR. The structure of NMe-(1,6)-CHA (**6**) contains two opposite βII-turns (highlighted in orange), whereas the two peptides **7** and **8** show both one βII′-turn (highlighted in blue) and one βVI-turn (highlighted in green).

groups, lipophilicity, the number of externally oriented NH groups or conformational homogeneity.[32] In a comprehensive NMR study, the three-dimensional conformational elements (highlighted in Figs. 1 and 4) explaining the high Caco-2 permeability were identified; intriguingly, they are also present in cyclosporin A (**1,** Fig. 1).[33]

In this NMR study 43 out of 54 peptides have two or more ^{1}H-NMR signal sets corresponding to two or more *cis-trans* isomers in equilibrium.[33] Only three (**6**, **7**, **8**) out of the eleven remaining conformationally homogeneous peptides show high permeability in the Caco-2 cell assay. Two template structures could be deduced that correlate to the high Caco-2 permeability. The first structure contains *N*Me(1,6)CHA (**6**), an all-*trans* peptide with two opposite β-turns. The second template structure is derived from the two peptides *N*Me(1,5)CHA (**7**) and *N*Me(1,2,4,5)CHA (**8**) which have similar backbone structures (Fig. 4). These contain two β-turns about D-Ala1-Ala2 and Ala4-Ala5; the *cis*-amide bond about Ala4-Ala5 results in a type VI β-turn. This emphasizes the need of an *N*-methylation in position 1 (***N*Me1**) within the second structure for Caco-2 permeability, whereas ***N*Me3** is not tolerated in the conformation and ***N*Me6** is distorting the template; a more detailed view is provided in the original manuscript.[33] Beck et al. compared those template structures with the 3D-structure of the bioavailable peptide cyclosporin A (**1**), published by Loosli et. al.[84] and Klages et al.[110], and with the 3D-structure of the tri-*N*-methylated somatostatin derivative **2,** published by Biron et. al.[29] The comparison with cyclosporin A (**1**) exhibited the similarity of the β-turns of the molecule (*N*Me-Gly3-*N*Me-Leu4 type II′ β-turn and D-Ala8-*N*Me-Leu9-*N*Me-Leu10-*N*Me-Val11 for the type VI β-turn, see also Fig. 1) with the second template structure.[33] The similarity between the somatostatin derivative **2** and *N*Me(1,2,4,5)CHA (**8**) is even more striking. The same *N*-methylation pattern (considering Pro as *N*Me-amino acid)[64] and positioning of the d–amino acid yielded nearly identical turn structures.[33]

A subsequent study by Marelli et al. indicates that the previously mentioned hypothesis regarding *N*-methylation of solvent

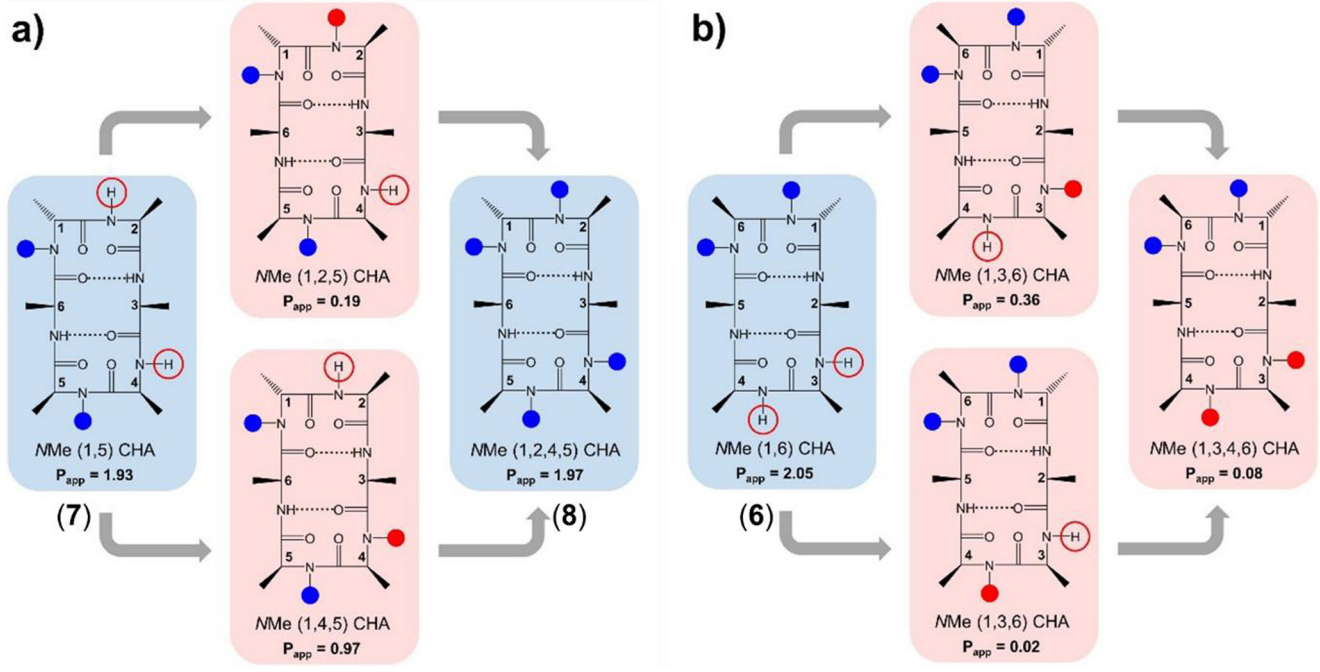


Fig. 5. Increasing methylation degree of CHAs: If one more solvent exposed NH group is methylated, the bioavailable di-*N*-methylated peptides *N*Me(1,5)CHA (**7**) and *N*Me (1,6)CHA (**6**) give non-bioavailable tri-*N*-methylated peptides. If all the solvent exposed NH groups are methylated, NMe(1,2,4,5)CHA (**8**; from **7**) is bioavailable. Reprinted with permission from Ref. 68; Copyright 2015 John Wiley and Sons.

exposed NH-groups only fits for a few permeable lipophilic peptides (PLPs).[68] Thus, *N*Me(1,5)CHA (**7**) compared to *N*Me(1,2,4,5)CHA (**8**) has two more solvent exposed NH-groups, but both show similar permeability in Caco-2 cell assays.[33,68] If another solvent exposed NH-group of peptide **7** is methylated, the two peptides obtained are no longer Caco–2 permeable (see Fig. 5***a***). In the case of the *N*Me(1,6)CHA (**6**), the tri- as well as the tetra-*N*-methylated derivative is not permeable in Caco-2 cell assays at all (see Fig. 5***b***).[68]

2.4.2. Enantiomeric studies of CHAs indicating carrier-mediated transport

For further proof of conformation-dependent or carrier-mediated transport of highly permeable peptides CHAs, *Marelli et. al.* examined the Caco-2 cell permeability of their enantiomers (**6***, **7***, **8*** and also **2***, **3***). Enantiomeric pairs have identical achiral physiochemical properties, but are expected to behave differently, if chiral biological transporter systems are involved.[68] The log$D_{7.4}$ and PAMPA values of these enantiomers are, of course identical, but the permeability in the Caco-2 cell assay for the CHAs **6**&**6***, **7**&**7*** and **8**&**8*** differ between the enantiomers. The permeabilities of PLPs **3**&**3*** in the Caco-2 cell assay differ by a factor of about 100 compared to the CHAs, implying that passive diffusion might be dominating and perhaps is accompanied by participation of an active transport. The observed similar permeability for the enantiomers supports this hypothesis.[68]

A second argument that proves a carrier-mediated transport is the differences between the values of the apical-to-basolateral and the basolateral-to-apical directions of the enantiomers. For an efflux transport the ratio (RO) of (b-a)/(a-b) should be above 1.5;[76] this was found for **6***, **7*** and **8**, whereas **6**, **7**, **8*** showed no indication for efflux transport. In conclusion this study showed that the spatial structure of peptides has a significant influence on their permeability behavior within carrier-mediated transport.[68]

2.5. Role of conformational flexibility of cyclic peptides on bioavailability

The conformational flexibility of molecules reduces the oral availability of drugs.[10] For the passive transport through tight junctions we may assume a multiple filtering function which require a resistance of a permeable conformation. When in the passage the conformation is changed the transport will be inhibited. Similarly, a transporter will also prefer a distinct conformation which should not alter during the transport. Hence, cyclic peptides, which exhibit a much more restricted conformational space than linear peptides, would be preferred candidates for orally available peptides. Under these considerations it is understandable that also *N*-methylated cyclic pentapeptides tend to be less permeable than cyclic hexapeptides.[69] The flexibility of cyclopeptides can be rationalized according to the *Dunitz-Waser* principle for cycloalkene.[111] This was explained by assuming a conformational equivalence of a cyclic peptide and the corresponding cycloalkene by substitution of each *trans* peptide bond by a *trans* alkene and a *cis* peptide bond by a *cis* carbon-carbon double bond. Cyclic hexapeptides, in this respect, correspond to cyclohexane whose chair conformation is less flexible than cyclopentanes (for more details see Ref. 112).

3. Conclusion

Peptides are excellent drug chemotypes, but development of orally bioavailable and stable biologically active derivatives is demanding. Enzymatic stability is engendered by using cyclic peptides and incorporating D-amino acids, but passage of peptides through the endothelial membrane of the gut is the remaining issue. Nature has evolved inspiring examples of peptides fulfilling these demands, such as cyclosporin A, but there are no simple guidelines for design of orally available peptides. This is because various uptake mechanisms are involved in the intestinal resorption process (passive transport, active transport, channels, involvement of tight junctions), and because of the inevitable effect of any chemical modification on bioactivity. In general, it may be safe to assume that highly lipophilic peptides (with high content on of lipophilic amino acids, such as Leu, and a low number of solvent exposed NH groups) will be preferentially transported by the passive mechanism. Permeability of more polar peptides – especially with functional groups to achieve the desired biological activity – seem to need a transporter associated with distinct conformational features. In practice, different mechanisms may work simultaneously and structural modifications can shift the preferred pathway.

Several structural parameters may be used to improve bioavailability, but those changes are often accompanied by additional unintended changes in the physicochemical properties of the compound. Despite this complexity, *N*-methylation of amide bonds – especially in cyclic peptides – has been identified as an important tool to improve the oral availability of cyclic peptides.

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