Organic &
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Chemistry

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Volume 14 | Number 1 | 7 January 2016 | Pages 1–372

ISSN 1477-0520

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ARTICLE

Synthesis and Conformational Analysis of Peptides Embodying 2,3-Methanopipecolic Acids

Luciano Ricci,[a] Lorenzo Sernissi,[a,b] Dina Scarpi,[a] Francesca Bianchini,[c] Alessandro Contini,[d] and Ernesto G. Occhiato[a]*

The conformational analysis of linear and cyclic peptides incorporating 2,3-methanopipecolic acids (or Cyclopropane Pipecolic Acids, CPAs) as conformationally constrained α-amino acids is reported. Compared to peptides containing proline or pipecolic acid, a striking increase of the *cis* isomer (42-92%) around the CPA amide bond is observed, both in water and organic solvent, when these unnatural amino acids are embodied in linear amino acid sequences. The rotational barrier around the same bond in water was calculated, resulting comparable to that for the prolyl *cis/trans* isomerization. In organic solvent, CPAs at the i+2 position of a peptide induce the formation of a type VIa β-turn secondary structure. When incorporated into a cyclic peptide, the *cis* geometry around the 2,3-methanopipecolic amide bond is still prevailing and, in the example studied herein (a cyclic RGD-containing ligand of $\alpha_v\beta_3$ integrin mimicking Cilengitide), conservation of the backbone geometry and side chain spatial orientation of the native peptide is also found. Given the importance of the proline *cis/trans* isomerism in many biological processes, CPAs could be useful as proline mimetics for probing protein-ligand interactions and generating novel bioactive compounds.

Introduction

Peptidomimetics are compounds designed to mimic the secondary structure of natural peptides or protein segments in order to attain enhanced activity and selectivity toward the biologic target.[1] The major issue in the rational design of peptidomimetics is the intrinsic flexibility of peptides which in most cases exist in numerous dynamically interconverting conformations. A strategy to lock the amino acid sequence into the active conformation is to incorporate some constraints and, to this end, a classic approach is to embody conformationally restricted building blocks like unnatural amino acids.[1] Among these, cyclopropane amino acids have been widely employed to reduce the conformational mobility in peptidomimetics[2-4] because of the rigidity and the partial unsaturated character of the three-membered ring.[5] For instance, rigid α-amino acids have been obtained by merging proline to a cyclopropane ring to form many 2,3-,[6] 3,4-[7] and 4,5-methanoprolines[8,9] which allowed for the synthesis of bioactive compounds and peptides with defined secondary

structure.[10]

This approach has been investigated less systematically with pipecolic acid, the six-membered ring homologue of proline, as only scattered examples of methanopipecolic acids (Figure 1a) are present in the literature.[8a,10h,11] On the other hand, pipecolic acids are components of a wide range of pharmacologically active compounds, including cyclopeptides,[12] and a more extensive exploration of the chemical space around methanopipecolic

(a) 2,3-, 4,5-, and 5,6-methanopipecolic acid derivatives
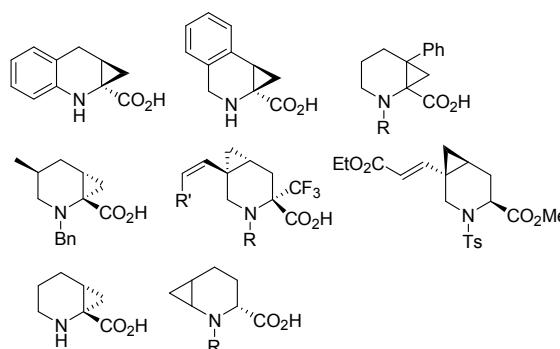
(b) (Poly)hydroxy-substituted 2,3-methanopipecolic acid derivatives
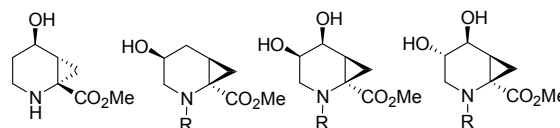
Figure 1. Selected examples of cyclopropane-fused pipecolic acid derivatives

[a] Department of Chemistry "U. Schiff", University of Florence, Via della Lastruccia 13, I-50019, Sesto Fiorentino, Italy.
[b] Current address: Department of Chemistry, Université of Montréal, PO box 6128, Station Centre-Ville, Montréal, QC, H3C 3J7, Canada.
[c] Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence, Viale Morgagni 50, I-50134, Florence, Italy.
[d] Department of Pharmaceutical Sciences, University of Milan, Via Venezian 21, I-20133 Milan, Italy.

acids could lead to the design of bioactive peptides with specific conformational preferences.

To this end we have recently reported the synthesis of a few (poly)hydroxy-substituted 2,3-methanopipecolic acids (which we called CPAs, cyclopropane pipecolic acids, Figure 1b),[13,14] as well as the corresponding 4- and 5-amino-substituted derivatives (Figure 2).[14] We have employed the latter as γ- and δ-amino acids to build RGD-containing cyclopeptides mimicking the loop of the natural $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrin ligands involved in the recognition,[15] and which displayed nanomolar activity towards both receptors.[14,16] However, to fully exploit the biomimetic potential of these constrained amino acids, their effects on the conformation of both linear and cyclic amino acid sequences in which they are embodied as α-amino acids had to be evaluated (Figure 2). For instance, it is known that the substitution of a pipecolic residue for a proline leads to a significant increase in the population of the *cis* conformer,[17] and that the *cis/trans* isomerism around the proline amide bond is often associated with important biological processes.[18] Therefore, the evaluation of how the *cis/trans* isomeric ratio is affected when 2,3-methanopipecolic acids replace a proline or a pipecolic acid is a key step toward the rational design of bioactive peptidomimetics.

In this paper we report on a new, simple approach for the stereodivergent synthesis of substituted CPAs, their incorporation in short linear and cyclic peptides, and a full conformational analysis of the latter in comparison, when appropriate, with amino acid sequences containing pipecolic acid and proline. A particular emphasis is given on the *cis/trans* isomerism around the 2,3-metanopipecolic peptide bond and the secondary structure of the linear and cyclic peptides in organic solvents and water.
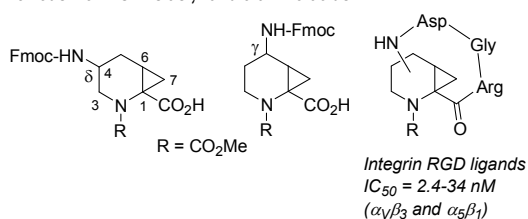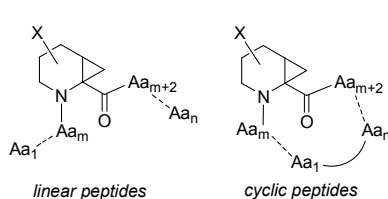

Figure 2. Inclusion of cyclopropane pipecolic acids (CPA) in linear and cyclic peptides

Results and discussion

Synthesis of CPAs and their incorporation in linear peptides

Since we wanted to evaluate the conformational effects of diastereomeric 2,3-methanopipecolic acids, a synthetic approach different from the OH-directed Simmons-Smith cyclopropanation was required.[14] Starting from known protected lactam **2** (Scheme 1),[19] α,β-unsaturated ester **5** was prepared in good yield via Pd-catalyzed methoxycarbonylation of lactam-derived enol phosphate **4**. It was consequently subjected to Corey-Chaykovsky cyclopropanation in DMSO with dimethylsulfoxonium methylide[20] which occurred with almost no facial selectivity and provided a 1.4:1 mixture of 2,3-methanopipecolic acid ester derivatives **6** and **7**. These could be easily separated by chromatography (40% and 35% yield, respectively), this approach thus allowing for the simultaneous preparation of both diastereomers in sufficient amounts (> 250 mg each in a single run from **2**) for the next peptide synthesis. Moreover, since *ent*-**2** can be prepared from the corresponding commercially available precursor [*R*-(−)-γ-hydroxymethyl-γ-butyrolactone], all possible stereoisomers of these hydroxy-substituted 2,3-methanopipecolic acid derivatives can be obtained.

Additionally, *N*-Cbz-protected (Cbz: benzyloxycarbonyl) compound **7** was easily converted into *N*-Boc-protected (Boc: *t*-butyloxycarbonyl) derivative **9** in two steps (Scheme 2), while starting from diastereomer **6** we tackled the protection of the amino group as *N*-9-fluorenylmethyloxycarbonyl (*N*-Fmoc) which is, besides *N*-Boc, the other protecting group of choice for peptide synthesis.

Concerning the structure of **6** and **7** (Figure 3), in compound **6** a NOE correlation observed between 4-H and the *endo* proton of the cyclopropane ring is in accordance with an axial orientation of 4-H and a *trans*-relative position between the OTIPS (triisopropylsilyloxy) and the cyclopropane ring (thus an *S* absolute configuration for the Cα atom). On the contrary, no NOE was observed between 4-H and the *endo* proton of the cyclopropane ring in compound **7**, demonstrating the *cis* relative position of the OTIPS and the cyclopropane ring (thus an *R* absolute configuration for the Cα atom). The low vicinal coupling value between 4-H and axial 3-H (1.6 Hz) is consistent with an axial orientation of the OTIPS group in **7**.
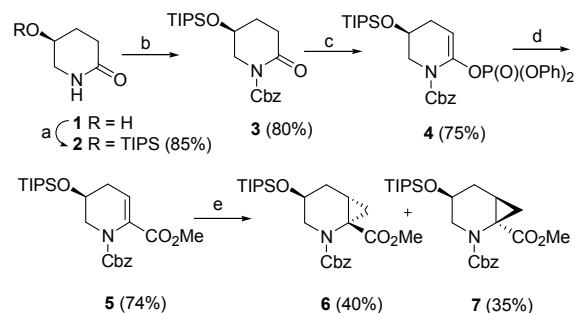
Scheme 1. Optimised synthesis of N-CO₂Bn protected 4-OTIPS-CPAs. Reagents and conditions: a) TIPSCl, imidazole, DMF, 35 °C; b) *n*-BuLi, CbzCl, THF, -78 °C; c) KHMDS, (PhO)₂P(O)Cl, THF, -78 °C; d) MeOH, CO, Pd(OAc)₂, Ph₃P, Et₃N, DMF, 60 °C; e) NaH, TMSOI, DMSO, 15 °C.

The synthesis of tri- and tetrapeptides embodying our CPAs is reported in Schemes 3, 4 and 5. Tripeptide **14** was prepared via dipeptide **13** starting from **8** as reported (Scheme 3).[14] Hydrolysis of the ester was carried out to remove the hydrophobic benzyl group, thus providing **15** which was eventually treated with 1.25 M HCl in anhydrous methanol to give methyl ester **16** as a water soluble HCl salt.

The corresponding diastereomeric compounds were prepared from **6** (Scheme 4), the CPA with *S* absolute configuration at the Cα atom. The coupling of **17** to *N*-Boc-protected alanine, carried out in THF in the presence of DEPBT [3-(diethoxyphosphoryloxy)-1,2,3-benzotri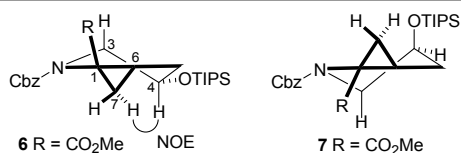azin-4(3*H*)-one] as the coupling reagent,[21] was complete in 3 days and provided Boc-Ala-CPA **18** in excellent yield (81%).[22] After ester hydrolysis, coupling of **19** with H-Gly-OBn was carried out as above and was complete in 4 days, furnishing tripeptide Boc-Ala-CPA-Gly-OBn **20** in 85% yield. The analysis of the ^{1}H NMR spectrum of **20** in CDCl$_3$ revealed a remarkably high amount of the *cis* isomer (see later) around the amide bond N-terminal to the CPA and a downfield chemical shift for the Gly NH proton (δ = 8.27 ppm) in this isomer, suggesting its possible participation to an intramolecular hydrogen bond. This particular observation prompted us to remove the N-protection from Ala (Scheme 5) and couple **22** with another amino acid (Cbz-Gly-OH) in order to have a tetrapeptide (**23**, 67% yield) in which the CPA was at the i + 2 position. Removal of the TIPS from both **20** (Scheme 4) and **23** (Scheme 5) provided the corresponding alcohols **21** (64%) and **24** (65%). Hydrogenation of **24** over 10% Pd/C in MeOH and TFA treatment provided water soluble peptide **25** as TFA salt in quantitative yield.
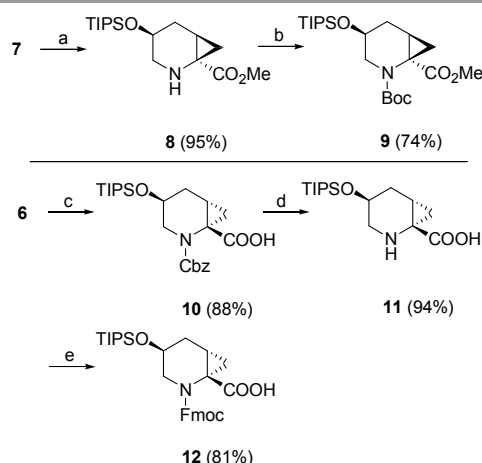
Conformational analysis of linear peptides incorporating CPAs

The identification of the *cis* and *trans* isomers in compounds **13**-**16** and **18**-**25** (Table 1) is possible by combined NOESY studies and analyses of the chemical shifts of the protons at C3 of the CPA moiety, in CDCl$_3$ or in D$_2$O as appropriate. Because of the magnetic anisotropy of the Ala carbonyl group, in all examined compounds the ^{1}H NMR signals of the axial and equatorial protons at C3 in the *cis* isomer are always more separated than in the *trans* isomer. In particular, in the *cis* isomers the axial 3-H is more upfield shifted (2.54-3.00 ppm) than in the *trans* isomers (2.93-3.43 ppm) and the equatorial 3-H is more downfield shifted (3.91-4.56 ppm *vs* 3.67-3.91 ppm) (Table S1, Electronic Supplementary Information). This assignment is confirmed in the *trans* isomers by the NOE cross-peak between the Ala H$_\alpha$ and the CPA 3-H$_{eq}$, whereas in the *cis* isomers a NOE cross-peak between the Ala methyl group and the *endo* proton of the cyclopropane ring (C7) is diagnostic.

It has been observed that peptides in which a pipecolic acid with *S* absolute configuration replaces a proline show an increase in the population of the *cis* isomer around the pipecolic amide bond. For example the *trans*/*cis* ratio in compound **28** (Figure 4 and Table 1) is 3:1, whereas the ratio is about 12:1 when Pro is in the same position (i+2) (**26**, Figure 4).[17] This increase has been attributed to the augmented steric interaction between the ε position of the pipecolic acid ring and the Cα substituent of the preceding residue.[17,23]

Scheme 3. Reagents and conditions: a) H$_2$ (1 atm), 10% Pd/C, EtOH, 25 °C, 23 h; b) 1.25 HCl in MeOH, 25 °C, 3 h

Scheme 2. Reagents and conditions: a) H$_2$ (1 atm), 10% Pd/C, EtOAc, 25 °C, 18 h; b) Boc$_2$O, Et$_3$N, MeOH, reflux, 2h. c) 1 N NaOH, MeOH, 50 °C, 24 h; d) H$_2$ (1 atm), 10% Pd/C, EtOAc, 25 °C, 18 h; e) FmocOSu, Na$_2$CO$_3$ aq., THF, 24 h.
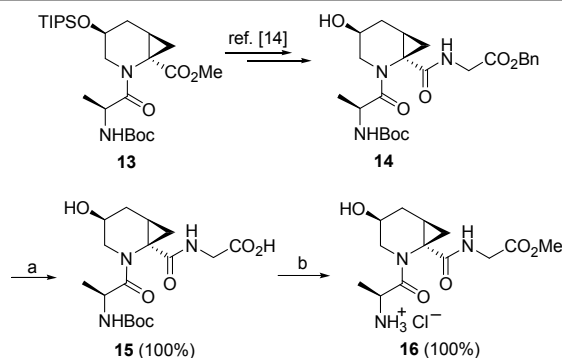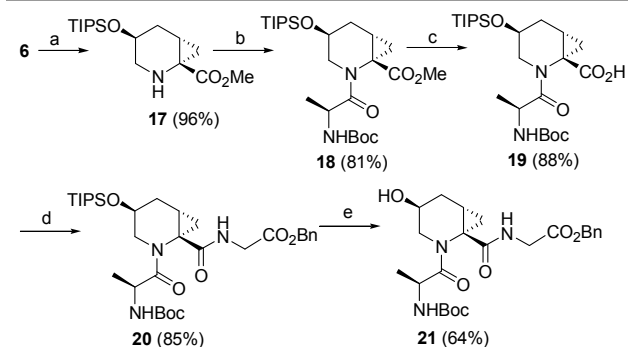

Scheme 4. Reagents and conditions: a) H$_2$ (1 atm), 10% Pd/C, EtOAc, 25 °C, 18 h; b) Boc-Ala-OH, DEPBT, DIPEA, THF, 35 °C, 3 d; c) NaOH, MeOH, 40 °C, 24 h; d) H-Gly-OBn hydrochloride, DEPBT, DIPEA, THF, 35 °C, 4 d; e) TBAF, THF, 25 °C, 2 h.

Figure 3. Stereochemical assignment for compounds **6** and **7**

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The tendency for a larger *cis* isomer population is even more marked when our 2,3-methanopipecolic acids are at a central (i+1 or i+2) or terminal position, with a ratio between the two conformers which is affected by the Cα absolute configuration of the embodied CPA. When CPA **8**, with 1*R* absolute configuration, is the terminal amino acid of a dipeptide sequence with N-Boc L-alanine (entry 4, compound **13**), the *cis* isomer is the most populated (about 69% of the *cis*/*trans* mixture) in CDCl₃.[24] The relative amount of the *cis* isomer decreases to 45% when the same CPA is the central amino acid of tripeptide Boc-Ala-CPA-Gly-OBn **14**. The *cis* relative amount only slightly changed in D₂O (43%) when the N-Boc protection was removed to give tripeptide **16** (entry 7). The formation of a weak H-bond between the Gly NH and the Ala carbonyl group to form a γ-turn could be invoked to explain the increase of the relative amount of the *trans* isomer of **14** in the organic solvent.[14] However, for both the *trans* and *cis* isomers of corresponding tripeptide **16** there is no experimental evidence of any turn- or H-bond-stabilized structure in water. In fact, variable temperature experiments in D₂O/H₂O 1:9 (from 20 to 55 °C) showed that no H-bonded structures exist, as the chemical shift temperature coefficients for Gly NH, measured at 500 MHz, are -8.57 and -8.0 ppb/K in the *cis* and *trans* form, respectively, which are in the range of amides not involved in H-bond (Figure S1a, ESI).
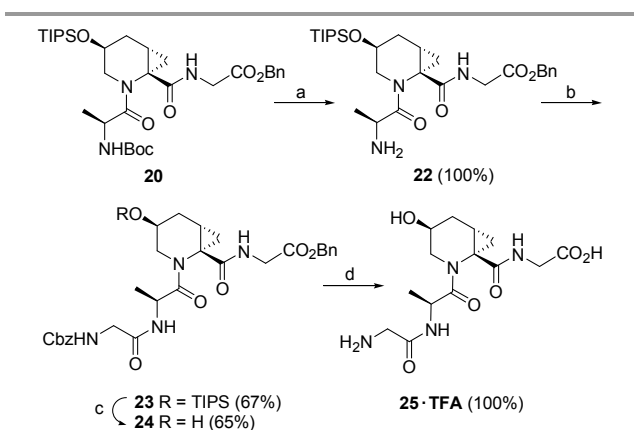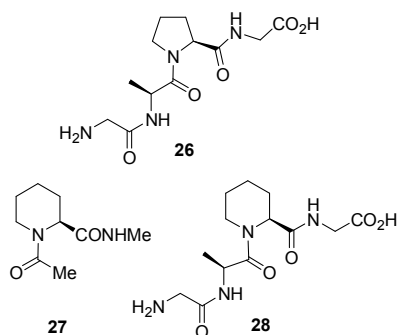
Interestingly, by increasing the temperature, we did not observe any sign of coalescence nor signal broadening for both the Gly NH and the protons of the CPA (e.g. 3-H$_{ax}$), with the chemical shift of the latter remaining practically unchanged during the experiments (Figure S1a, ESI). Moreover, there was no change in the *cis*/*trans* ratio when increasing the temperature from 20 to 55 °C. These results strongly indicated a high rotational barrier between the *cis* and *trans* isomers around the CPA amide bond in **16**, but its evaluation in water was not possible.[25] A coalescence temperature of 80 °C at 400 MHz, corresponding to a rotational barrier $\Delta G^{\ddagger}$ = 17.8 kcal/mol, has been instead measured in water for pipecolic acid derivative **27** (Figure 4).[23] The rotational barrier of **16** was thus computationally evaluated by umbrella sampling followed by a potential of mean force (PMF) analysis,[26] in comparison with the same peptide embodying a pipecolic acid (**m 16**) (Figure 5, see also ESI for additional details).

For the CPA derivative **16**, a barrier of 19.2 kcal/mol was obtained, whereas a barrier of about 1 kcal/mol lower was obtained for the corresponding peptide with pipecolic acid. Thus, the presence of the cyclopropane ring in CPA not only causes an increase of the *cis* isomer population (Table 1), but it also determines an increase of the rotational barrier, bringing it closer to that measured for the prolyl *cis*/*trans* isomerization.[27]

When CPA **11**, having 1*S* absolute configuration at the Cα, is the terminal amino acid of the Boc-Ala-CPA dipeptide, the relative amount of the *cis* isomer in CDCl₃ also increases compared to the corresponding peptides containing pipecolic acid, as found for both methyl ester **18** (entry 8) and free acid **19** (entry 9) (60 and 50%,

Scheme 5. Reagents and conditions: a) Sn(OTf)₂, DIPEA, DCM, 25 °C, 2 h; b) Cbz-Gly-OH, DEPBT, DIPEA, THF, 35 °C, 4 d; c) TBAF, THF, 25 °C, 2 h; d) H₂, 10% Pd/C, MeOH, 25 °C, 3 h, then TFA.

Figure 4. Simple amino acid sequences embodying proline and pipecolic acid

Table 1. Relative amount of *cis* isomer in the studied compounds

Entry	Compd	Cα config.	R	R'	R''	Solvent	% cis
1	**26**	-	-	-	-	D₂O	8
2	**27**	-	-	-	-	D₂O	28
3	**28**	-	-	-	-	D₂O	25
4	**13**	*R*	TIPS	OMe	Boc	CDCl₃	69
5	**14**	*R*	OH	Gly-OBn	Boc	CDCl₃	45
6	**15**	*R*	OH	Gly-OH	Boc	CD₃OD	25
7	**16**	*R*	OH	Gly-OMe	H	D₂O	43
8	**18**	*S*	TIPS	OMe	Boc	CDCl₃	60
9	**19**	*S*	TIPS	OH	Boc	CDCl₃	50
10	**20**	*S*	TIPS	Gly-OBn	Boc	CDCl₃	92
11	**21**	*S*	OH	Gly-OBn	Boc	CDCl₃	80
12	**22**	*S*	TIPS	Gly-OBn	H	CDCl₃	88
13	**23**	*S*	TIPS	Gly-OBn	Cbz-Gly	CDCl₃	86
14	**24**	*S*	OH	Gly-OBn	Cbz-Gly	CDCl₃	79
15	**25**	*S*	OH	Gly-OH	H-Gly	D₂O	42

respectively). Diminished steric repulsions between the Ala side chain and the Cα position of the CPA, as a consequence of the replacement of the pipecolic acid C_{sp3}-H bond with a cyclopropane C-C bond pointing away from the preceding amino acid side chain, could explain the increase of the *cis* isomer molar fraction in CPA-embodying peptidomimetics.[28] When **11** is central in a sequence, as in tripeptide **20** (entry 10), the *cis/trans* ratio is even more markedly shifted toward the *cis* isomer which becomes predominant (92%) in CDCl$_3$. This also occurs with the corresponding 4-OH substituted tripeptide **21** (entry 11) although the relative amount of the *cis* isomer is lower (80%).

Detailed solution ^{1}H NMR studies on both peptides **20** and **21** in CDCl$_3$ revealed the presence of a type VI β-turn-like structure involving the *cis* isomers. The type VI β-turn is a relatively uncommon protein secondary structure which involves a *cis* peptide bond N-terminal to a L-proline residue situated at the *i* + 2 position.[29] The H-bond between the NH of the fourth amino acid (i+3) and the carbonyl group of the first amino acid specifically characterizes a type VIa β-turn. In our peptides, if the Boc carbonyl group is thought as the carbonyl group of the *i* amino acid in the sequence, then the CPA occupies the *i* + 2 position and the stage is set for the organization of this secondary structure. In both tripeptides, the *cis* conformation of the major isomer was demonstrated by the NOE between the methyl group of the alanine and the *endo* 7-H proton (Figure 6). Moreover, in the ^{1}H NMR spectrum the signals of 3-H$_{ax}$ and 3-H$_{eq}$ in the major isomer are more separated (Table S1) than in the *trans* isomer, confirming this assignment. A NOE between Gly NH and Ala Cα-H suggests that Gly NH is correctly orientated to possibly form an H-bond with the Boc carbonyl group, which would nucleate the 10-membered type VIa β-turn structure. Consistently, in the *cis* isomer the $^3J_{NH,CH\alpha}$ coupling constant of the alanine residue is 4.8 Hz (in the *trans* isomer is much larger, 8.0 Hz), corresponding to a dihedral angle of about 125° between the two protons, thus indicating that a substantial fraction of the *cis* isomer adopts a folded secondary structure.
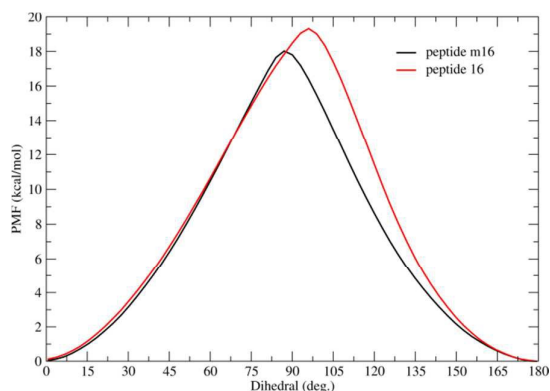
Figure 5. Energy barrier calculated for the *cis-trans* isomerization of the amide bond connecting Ala and CPA (peptide **16**) and that of a model peptide where CPA is replaced by the corresponding pipecolic acid (peptide **m 16**).
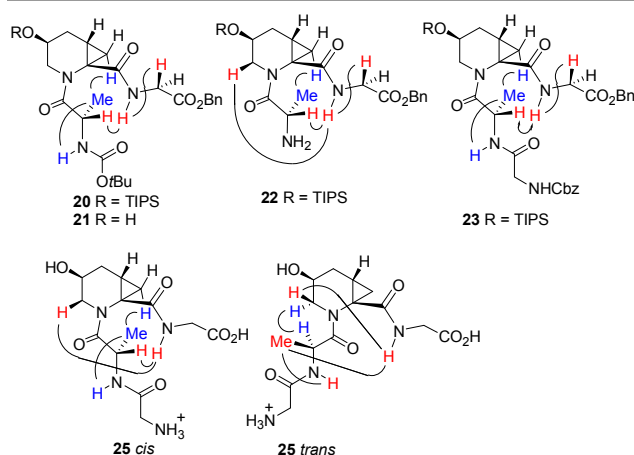
Figure 6. NOE correlations in compounds **20**-**23** and **25**.

The occurrence of the H-bond between Gly NH and the Boc C=O group is further suggested by the markedly deshielded (8.28 and 8.37 ppm) signals of the Gly NH proton in the *cis* isomer of both compounds (in the *trans* isomers it resonates at 6.47 and 6.57 ppm). Very interestingly, the *cis/trans* ratio changed just a little upon removal of the Boc group to give **22**, which clearly demonstrates that it is the presence of the CPA scaffold that mainly causes the predominance of the *cis* isomer in the organic solvent. In the major isomer of **22** (in a 7:1 ratio with the minor isomer) the methyl group of Ala has a NOE cross-peak with the *endo* 7-H proton (Figure 6) which is consistent with a *cis* geometry. Gly NH now resonates more upfield shifted (at 7.03 ppm), being not engaged in H-bonding, but still shows a NOE cross-peaks with Ala Hα. Moreover, Gly NH shows a NOE also with 3-H$_{ax}$ which suggests a downward rotation of the CPA carbonyl group toward a favoured bisected conformation of the cyclopropane ring.[30]

These observations, together with the slightly decrease in the *cis/trans* ratio compared to **20**, suggest that the H-bond should actually contribute to a further stabilization of the *cis* isomer in the latter compound. To ascertain that the same secondary structure is present in a true tetrapeptide with CPA **11** at the i + 2 position, we studied compounds **23** and **24** in CDCl$_3$, and **25** in water. The ^{1}H NMR analysis of tetrapeptides **23** and **24**, which were obtained as 6:1 and 3.8:1 mixtures, respectively, of *cis* and *trans* isomers (Table 1), confirmed the presence of the type VIa β-turn secondary structure in CDCl$_3$ for the major (*cis*) isomer (Figure 6). First, the $^3J_{NH,CH\alpha}$ coupling constant of the alanine residue in the *cis* isomer (5.2 Hz for both compounds) suggests that the *cis* isomer mostly adopts a folded secondary structure. Then, the pattern of NOE correlations was the same than in compounds **20** and **21** and stronger evidences of an H-bond between NH (i+3) and C=O (i) were found, at least for **23**. The larger Δδ between 3-H$_{ax}$ and 3-H$_{eq}$ (1.6-1.85 ppm) (Table S1), the NOE between the alanine methyl group and the *endo* 7-H proton of the cyclopropane ring and the NOE cross-peak between Ala Hα and the amide proton of Gly (i+3) (Figure 6) in the major isomer are in accordance with the *cis* conformation of the CPA amide bond. The downfield chemical shift of Gly

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(i+3) NH (δ = 7.91 ppm in **23**) suggests its participation in the intramolecular H-bonding with the carbonyl group of Gly (i). A solvent titration study carried out by adding increasing amounts of DMSO-d_6 (from 1.6 to 23% v/v) to a 9 mM solution of **23** in CDCl$_3$ was conclusive about the formation of such H-bond. In fact, we observed a very small variation of Gly (i+3) NH chemical shift (Figure 7) in the major isomer, whereas the other amide protons were markedly downfield shifted upon increasing the relative amount of DMSO-d_6. The H-bond between Gly (i+3) NH and Gly (i) C=O becomes weaker when TIPS protection is removed from **23**, as in compound **24** Gly (i+3) NH resonate more upfield shifted (7.63 ppm). Interestingly, removal of the large TIPS protecting group from **20** and **23** caused an increase of the *trans* isomer molar fraction in both alcohols **21** and **24**. The *trans* isomers of **20** and **23** should reasonably be less favoured because of the presence of the large group at C4 which could cause unfavourable steric interactions with Ala side chain and CHα.

In compound **24**, the Ala (i+1) NH resonates more downfield at 7.17 ppm (at 6.41 ppm in **23**) suggesting some H-bonding involving this proton (e.g. with the Cbz C=O group). A conformational search carried out on **24** and including NOE derived restraints (Table S2, ESI) resulted in nine conformations in a 2 kcal/mol interval, with the global minimum conformer showing a H-bond between the Cbz C=O group and Ala NH, but not the one between Gly (i) C=O and Gly (i+3) NH. The latter is instead present in an energetically higher conformation (ΔE = 1.6 kcal/mol) to give the type VIa β-turn structure that was anticipated by the ^{1}H NMR studies (Figure S3, ESI).

The ^{1}H NMR analysis of tetrapeptide **25** in water (10 mM solution in D$_2$O/H$_2$O, 1 : 9) revealed a greater separation of the 3-H$_{ax}$ and 3-H$_{eq}$ chemical shifts in the minor isomer ($\Delta\delta$ = 1.37 ppm *vs.* 0.76 in the major isomer, Table S1) and a NOE between Ala H$_\alpha$ and 3-H$_{eq}$ in the major isomer. This allowed us to conclude that in this solvent there is a marked decrease of the relative amount of the *cis* isomer (42%, Table 1), although its molar fraction is still greater than that of the *cis* isomer in the corresponding tetrapeptide **28** (25% in water) embodying a pipecolic acid in the same position.
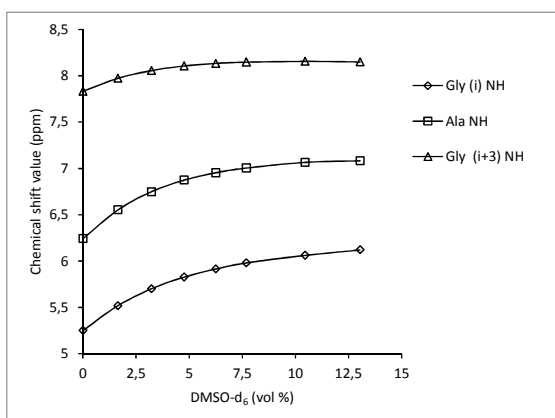
The NOE pattern in the *cis* isomer of **25** (Figure 6) is the same observed in organic solvent for compound **23**. In fact, cross-peaks between the Ala methyl group and the *endo* 7-H proton, between Gly (i+3) NH and Ala Hα, and most important, between Gly (i+3) NH and 3-H$_{ax}$, suggest that a substantial fraction of the conformers possessing the *cis* CPA amide bond adopts a geometry with the cyclopropane ring bisected by the CPA carbonyl group [with the consequent lack of H-bonding between Gly (i+3) NH and Gly (i) C=O]. The same NOE between Gly (i+3) NH and 3-H$_{ax}$ is present in the *trans* isomer (Figure 6), for which we also observe a cross-peak between Gly (i+3) NH and the Ala methyl group. Variable temperature experiments (from 20 to 65 °C) carried out on **25** in water at 500 MHz resulted in large $\Delta\delta/\Delta T$ for all amide protons (from -6.57 to -7.71 ppb/K, Figure S1b) in both rotamers as none of them is engaged in H bonding. Interestingly, the *cis*/*trans* ratio in water seems independent on the absolute configuration of the CPA embodied in the peptide, as the same ratio was observed in peptide **16** embodying a (1*R*)-CPA. Similarly to compound **16**, in **25** no sign of coalescence was observed while increasing the temperature for both the amide and CPA protons of **25**, the chemical shift of the latter remaining unchanged during the experiments. Moreover, there was no change in the *cis*/*trans* ratio when increasing the temperature from 20 to 65 °C. Again, these results were strongly indicative of a high rotational barrier in water between the *cis* and *trans* isomers around the CPA amide bond.[27]

#1 (50.7%)   #2 (23.2%)

#3 (19.8%)   #4 (5.9%)

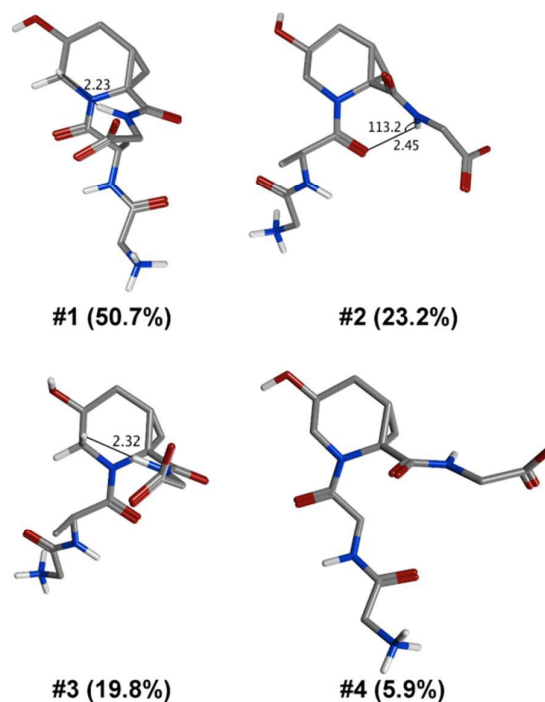**Figure 8.** Representative conformations of the four principal clusters obtained from the analysis of the 300 K trajectory obtained by REMD simulations on peptide **25**. Populations are referred to the analysis of the 300 − 400 ns. Selected distances are reported in Å, angles in degrees.

Figure 7. DMSO-d_6 titration study of compound **23** in CDCl$_3$.

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Replica Exchange Molecular Dynamic (REMD) simulations were performed on peptide **25** by adapting a protocol previously used to simulate the folding of short peptides bearing non-natural amino acids in their sequence.[31] The REMD trajectory obtained at 300 K was then analyzed by clustering, providing the four main conformations depicted in Figure 8.

Two conformations present a *cis* geometry around the CPA amide bond (clusters #1 and #4, having populations of 50.7 and 5.9%) while the other two show *a trans* geometry (#2 and #3, having populations of 23.2 and 19.8%). The two components of each *cis* or *trans* pair differ by the orientation of the amino acidic carbonyl of the CPA residue, that in one case points "upward" (conformations #2 and #4). Compared to the experimental values, the overall *cis* population (56.6%) appears overestimated, but the principal geometry fits well with NOE findings. In fact, in the most populated *cis* isomer (conformation #1, Figure 8), the Gly (i+3) NH has a distance to 3-H_{ax} of 2.23 Å, compatible with the NOE observed. A similar distance (2.32 Å) was measured for the minor *trans* conformer (#3, Figure 8), in accordance with the experimental NOE. The *trans* conformation #2 presents a distance between the Gly (i+3) NH and the Ala C=O group of 2.45 Å, which might suggest the presence of an H-bond, although not observed experimentally. However, a N-H···O angle of 113.2° indicates that such an interaction, if it exists,[32] is very weak and cannot by itself stabilize the γ-turn conformation, in line with the NMR results.

Synthesis of a cyclopeptide embodying a CPA

To evaluate the *cis*/*trans* isomerism around the CPA amide bond in a cyclic peptide and the conformational changes undergone by the latter, because of our interest in integrin receptor ligands[14,16] we decided to include CPA **8** into a cyclopeptide analogous to Cilengitide.[33] This is a known $\alpha_V\beta_3$ receptor antagonist which has in its sequence Arg, Gly, Asp and the unnatural D-Phe and N(Me) Val amino acids.[34] CPA **8** was pictured to substitute the latter amino acid, as the remaining sequence D-Phe-Asp-Gly-Arg is responsible for the recognition.[33b] The synthesis in solution of cyclopeptide **35** is reported in Scheme 6 and started with the coupling of CPA **8** to Boc-D-Phe-OH in the presence of DEPBT as the coupling reagent. The coupling proceeded smoothly, although it required the usual long time (4 days) to be complete, providing **29** in 75% yield after chromatography as a mixture of *cis* (45%) and *trans* isomer (55%) around the CPA amide bond. After hydrolysis of the methyl ester group, the coupling with dipeptide H-Arg(Mtr)-Gly-OBn was similarly uneventful, providing tetrapeptide **31** (molecular ion at *m/z* 1076 [M+ + 1]) in 77% yield as a 1.4:1 mixture of rotamers. Finally, after quantitative deprotection of D-Phe amino group to give **32**, further coupling with Cbz-Asp(*t*-Bu)-OH gave fully protected pentapeptide **33** (molecular ion at *m/z* 1281 [M+ + 1]) in 75% yield. Hydrogenation of **33** over 10% Pd/C then provided **34** with the two unprotected amino and carboxylic group. The cyclization of this intermediate was carried out in a dilute (3.5 mM) solution in anhydrous THF by using DEPBT as the coupling
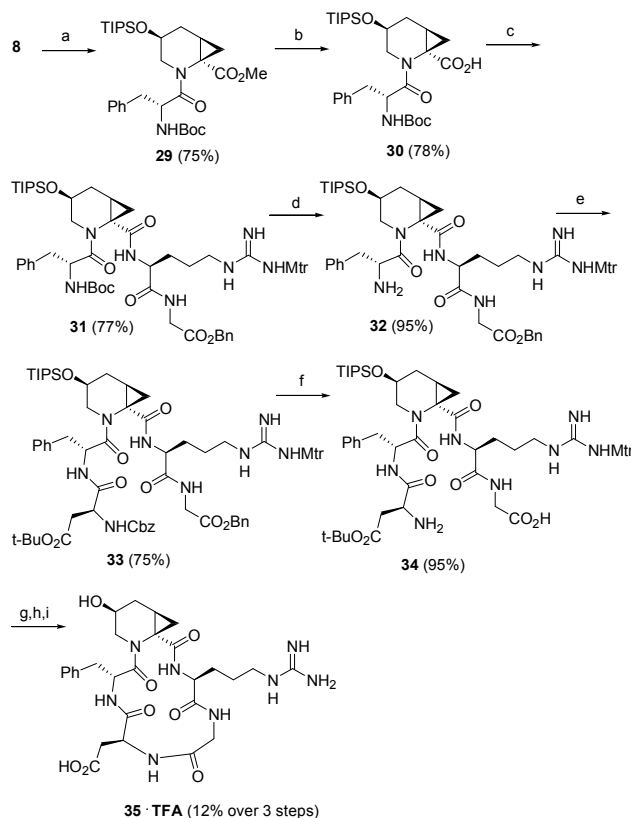

Scheme 6. Reagents and conditions: a) DEPBT, DIPEA, Boc-D-Phe-OH, THF, 35 °C, 4 d; b) 1 N NaOH, MeOH, 25 °C, 3 d; c) DEPBT, DIPEA, H-Arg(Mtr)-Gly-OBn, THF, 35 °C, 4 d; d) Sn(OTf)₂, CH₂Cl₂, 25 °C, 28 h; e) DEPBT, DIPEA, Z-Asp(OtBu)-OH, THF, 35 °C, 4 d; f) H₂, 10% Pd/C, EtOH, 25 °C, 18 h; g) DEPBT, DIPEA, THF, 35 °C, 4 d; h) TBAF, THF, 25 °C, 4 h; i) TFA/TIS/H₂O 95:2.5:2.5, 25 °C, 18 h.

reagent, which furnished, after exhaustive deprotection, macrocycle **35** (molecular ion at *m/z* 615 [M+ + 1]) in 12% yield over three steps and after HPLC purification (94% purity).

Conformational analysis of CPA-embodying cyclopeptide 35

The structure and connectivity of peptidomimetic **35** was unambiguously assigned by means of mono- and bidimensional ¹H NMR spectroscopy in aqueous solution (all ¹H NMR data are reported in Table S3 in ESI) which revealed the presence of a major species (86%) we could ascribe to the *cis* isomer around the CPA amide bond. The resonance separation of the 3-H protons (Δδ = 0.58 ppm in D₂O and 0.77 ppm in CD₃OD) is in fact very similar to that observed in the *cis* isomer of compound **29** and, moreover, we did not find any NOE cross-peak between the CHα of D-Phe and those at C3 of the CPA. Instead, a NOE cross-peak between the CHα of D-Phe and the *endo* proton (7-H) of the cyclopropane ring confirms the *cis* geometry (Figure 9a). A weak NOE is also found between the *ortho* protons of the D-Phe phenyl ring and the *endo* 7-H. Most data suggest the existence of a preferred conformation for ligand **35**, even though the temperature coefficient values between -4.28 and -7.1 ppb/K (Figure S6, ESI) indicate that none of the N-H protons is tightly locked in an intramolecular H-bonded state.

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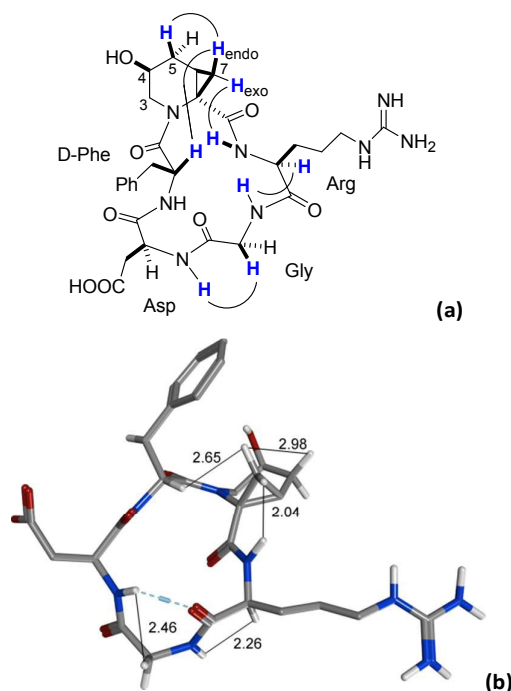
Figure 9. (a) NOE correlations in cyclic peptide **35**. (b) Representative geometry of the most populated cluster obtained by REMD simulation on cyclopeptide **35**, followed by cluster analysis of the last 50 ns of the 300K trajectory. Distances corresponding to experimental NOEs are shown.

The evaluation of the NOE contacts showed the presence of two medium-strong sequential CHα(i)/NH(i+1) cross-peaks along the Arg-Gly-Asp sequence, whereas such contact could not be found between the CHα of Asp and the N-H of D-Phe. A strong NOE contact, very useful for the determination of the putative preferred conformation in solution, was found between the N-H of Arg and the *exo* proton (7-H) of the cyclopropane ring, suggesting an orientation of the N-H bond toward the small ring (Figure 9a). Given the "upward" preferred orientation of Arg N-H toward the 7-H, the existence of a NOE cross-peak between the N-H of Gly and the CHα of Arg, indicates that Gly N-H points "downward", with the Arg carbonyl group instead pointing "upward" to form a γ-turn with Asp N-H. This is corroborated by the relatively low chemical shift value of Asp N-H (7.97 ppm), the relatively high $^3J_{NH-\alpha H}$ coupling constant (9 Hz), as well as the lowest temperature coefficient (-4.28 ppb/K) for this amide proton. However, Gly N-H does not seem engaged in H-bonds (e.g. in a possible γ-turn with the carbonyl group of CPA) as the high chemical shift value (8.77 ppm) and temperature dependence (-7.1 ppb/K) suggest. As a further indication of the existence of a preferred conformation in aqueous solution, the two diastereotopic CHα of Gly resonate as highly separated dds (4.19 and 3.37 ppm) and, moreover, only one of them (i.e. that at 4.19 ppm) shows a cross-peak with the N-H of Asp.

REMD simulations on compound **35**, followed by cluster analysis of the 300K trajectory, resulted in three main conformations (Figure S4, ESI), approximately in a 65:20:14

ratio and all with *cis* configuration at the CPA amide bond (four other conformations have collectively totaled the 1% of the overall population). The spatial orientation of the NH and C=O groups in the most populated conformation (Figure 9b), as well as the measured distances, perfectly matches with the NOE correlations found in **35**. The Arg N-H actually points towards the *exo* proton (7-H) of the cyclopropane ring allowing an energetically favored bisected conformation of the cyclopropane ring with the CPA carbonyl group.[30] Moreover, the analysis of the most representative conformer of **35** indicates the formation of the γ-turn between the Arg carbonyl group and Asp NH (H···O distance = 2.10 Å, N-H···O angle = 139.3°) as predicted by the NMR analysis.

Interestingly, a comparison of the reported preferred conformation of Cilengitide in water[33b] with that of ligand **35** resulted in the same relative orientation of the side chains of D-Phe, Asp and Arg, as well as of the N-H and CO groups of the latter. The γ-turn between the Arg carbonyl group and Asp NH residue in Cilengitide was also present in **35**. The superimposition of the most representative geometry of **35** with that of Cilengitide co-crystallized with the extracellular segment of integrin $\alpha_V\beta_3$,[35a] resulted in a RMSD = 1.07 Å evaluated on the backbone atoms, with a good match of the side chains (Figure S5, ESI). The calculated distance between the β carbon atoms of Asp and Arg residues (7.8 Å), an important parameter for $\alpha_V\beta_3$ affinity, was slightly shorter than that in Cilengitide (8.9 Å).[35] Although out of the scope of this work, compound **35** was tested on M21 human melanoma cells expressing high levels of $\alpha_V\beta_3$ heterodimer and actually showed capacity to inhibit binding of the cells to vitronectin (IC$_{50}$ = 150 ± 50 nM), but less than Cilengitide (3.8 ± 1.7 nM) (Figure S8, ESI).[36]

Conclusions

In conclusion, 2,3-methanopipecolic acids (herein referred Cyclopropane Pipecolic Acids, CPAs) are conformationally constrained α-amino acids which can be incorporated into amino acid sequences to build linear and cyclic peptidomimetics. The coupling of the CPA N-terminus to other amino acids is much slower if compared to proline (3-4 days *vs.* 4-6 h) under the best conditions, but nevertheless it provides the target peptide in excellent yield. A thorough conformational analysis of the latter allowed us to conclude that when embodied in short (three to four) amino acid sequences, the presence of a cyclopropane pipecolic acid determines a noticeably increase of the *cis* isomer around the CPA amide bond, whose relative amount (42-92%) in both water and organic solvent is always and markedly higher than in the corresponding peptides containing a simple pipecolic acid or a proline. In organic solvent (CDCl$_3$), the *cis/trans* isomer ratio depends on the absolute configuration of the CPA Cα atom, with the *cis* isomer becoming predominant (79-92%) in tri- and tetrapeptides embodying a CPA with 1*S* absolute configuration. In this solvent, when (1*S*)-CPA is in the i+2 position, it forces the peptide to fold into a type VIa β-turn secondary structure. Instead, in water, the relative amount of

the *cis* isomer decreases to 42-43% irrespective of the Cα absolute configuration of the used 2,3-methanopipecolic acid, with the loss of the secondary structure found in CDCl₃. Finally, in these short peptides, the calculated rotational barrier around the CPA amide bond (19.2 kcal/mol) in water is larger than that measured or calculated for the corresponding peptides embodying a simple pipecolic acid. CPAs are suitable to be incorporated as α-amino acids in cyclic peptides, too. As an example, the inclusion of a CPA for a N(Me)Val in the amino acid sequence of Cilengitide generated a cyclic peptidomimetic, in which the *cis* isomer was still predominant, with low RMSD (1.07 Å) on backbone atoms and similarly oriented side chains.

Because of their features, in particular the preferential *cis* geometry and the high rotational barrier around the CPA amide bond, cyclopropane pipecolic acids are suitable to be incorporated in peptidomimetics as constrained α-amino acids as tools for probing protein-ligand interactions and generating novel bioactive compounds.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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linear peptides   R = Cbz, Boc, Fmoc
(all four stereoisomers)   *cyclic peptides*
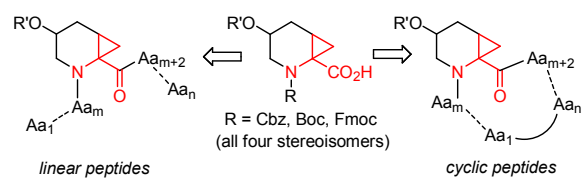
When 2,3-methanopipecolic acids replace a proline in peptides, a marked preference (42-92%) for the *cis* geometry around the pipecolic amide bond is observed in both water and organic solvent.