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Structural properties of cyclic peptides containing *cis*- or *trans*-2-aminocyclohexane carboxylic acid †

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A series of cyclic peptides containing either *cis*- or *trans*-2-aminocyclohexane carboxylic acid as mimics for L-proline has been synthesized and their structural properties have been investigated using NMR and MD methods.

Introduction

The secondary structure of a protein is mainly characterized by ordered conformations of periodic nature (helix, sheet), stabilized by hydrogen bonds between donors (NH) and acceptors (C=O) of peptide bonds. A polypeptide chain cannot fold into a compact globular structure without an inversion of the chain direction involving tight turns. Usually these occur on the exposed surface of proteins. Consequently, the characteristic secondary structure element of a surface exposed loop is the reverse turn. Protein–protein interactions often rely on the recognition of relatively small epitopes present in such loop structures on the surface. Therefore, turns are important for this type of supramolecular interactions. The three-dimensional topography of protein loop structures can be reproduced by small peptides, which may be utilized in the design of small molecule antagonists of protein–protein interaction in drug development.

Turns have been classified according to the number of amino acid residues involved as γ-turns (three amino acids), β-turns (four amino acids), α-turns (five amino acids), or π-turns (six amino acids).[1] Extensive knowledge exists on the conformational preferences of proteinogenic amino acids. Some types of turns are stabilized by special amino acids. It is well known that substitution of one L- by a D-amino acid or incorporation of proline causes strong conformational bias.[2] The pyrrolidine ring in L-proline constrains the dihedral angle ϕ to $-60°$. Therefore, proline with a *trans* configured peptide bond is found preferentially in position $i + 1$ of a βI- or βII-turn.

We recently expanded the repertoire of building blocks with reproducible bias on the conformation of native, bioactive peptides by β-amino acids. Incorporation of a distinct β-amino acid in cyclic RGD peptides results in the stabilisation of the overall secondary structure.[3] It was also shown by several groups that linear peptides exclusively composed of β-amino acids (β-peptides) form a number of stable secondary structures such as sheets and helices.[4] β-Amino acids contain one additional carbon atom between C^{α} and N. Hence, the peptide chain is elongated by one skeleton atom and one additional torsion angle (μ) is present. β-Amino acids are not as common in nature as their α-analogues. Some β-amino acids are found in natural cyclic peptides such as leualacin or in astins which adopt well-defined secondary structures.[5]

Pavone *et al*. reported on cyclic tetra-, penta- and hexapeptides with two β-Ala residues located in the positions i or $i + 3$ of β-turns. However, this conformation may be mainly caused by the peptide sequences.[6] Besides that, β-amino acids have so far not been found in well-defined positions of β-turns. Kessler and coworkers designed carbohydrate type β-amino acids which show the ability to constrain linear backbone conformations or form distinct β- or γ-turn structures in several different peptides such as somatostatin or Leu-enkephalin analogues.[7] It has been shown that peptides containing β-homo-aminoacids frequently exhibit retarded metabolism and are sometimes stable against amino peptidases.[8] Moreover, Raines and coworkers incorporated β-amino acids into an enzyme to replace an existing β-turn with improved conformational stability.[9]

Consequently, β-amino acids have potential as inducers of secondary structure and even single β-amino acid residues stabilize discrete conformations in cyclic peptides. The special role of 2-aminocycloalkane carboxylic acids in this context has not yet been investigated. 2-Aminocycloalkane carboxylic acids, forming a subclass of β-amino acids, have meanwhile found broad application in chemistry and peptide synthesis.[10] Gellman *et al*. described several linear peptides containing *trans*-2-aminocycloalkane carboxylic acids which form 12- and 14-helices.[11] Recently, investigations on linear peptides with alternating sequence of α-amino acids and 2-aminocycloalkane carboxylic acids have been made.[12]

We embarked on a project to reveal the influence of a single 2-aminocyclohexane carboxylic acid (ACHC), either in the *cis*- or *trans*-form on the conformation of cyclic peptides, in order to eventually establish general principles for the application of these building blocks in the design of conformationally homogeneous cyclic peptides for the spatial screening approach and drug design.

Results and discussion

Here we report on our investigations regarding the solution structure of cyclic penta- and hexapeptides containing a single 2-aminocyclohexane carboxylic acid. The three-dimensional structures of the peptides were determined in solution by NMR and molecular dynamics simulations. 2-Aminocyclohexane carboxylic acids, where the torsion angle μ is constrained by cyclization, were tested as mimics for the proline residue in these peptides. In order to complement the structural data with preliminary information on a potential biological response, the peptide sequences were designed to contain the C-terminal part IDSPLN or DSPLN, resp., of the putative protein–protein recognition sequence TQIDSPLN.

The TQIDSPLN sequence is present in vascular cell adhesion molecule-1 (VCAM-1), expressed on epithelial cells during inflammatory processes.[13] VCAM-1 binds to the integrin $\alpha_4\beta_1$ (very late antigen-4, VLA-4),[14] a heterodimeric transmembrane glycoprotein receptor expressed on lymphocytes, monocytes, eosinophils, basophils, and mast cells. The integrin has been detected on neutrophils in certain inflammatory settings. The interaction between VLA-4 and its ligands is supposed to be

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/ob/b3/b312432k/

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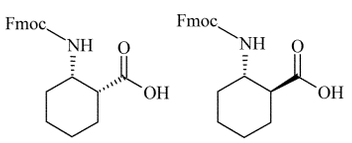
Table 1 Synthesized peptides

No.	Sequence
P1	c-(-Ser-cACHC-Leu-Asp-Asn-)
P2	c-(-Ser-tACHC-Leu-Asp-Asn-)
P3	c-(-Ser-cACHC-Leu-Asp-Ile-Asn-)
P4	c-(-Ser-tACHC-Leu-Asp-Ile-Asn-)

necessary for leukocyte adhesion, migration and activation both in normal immune response as well as in the progression of various inflammatory and autoimmune diseases such as asthma, contact hypersensitivity, multiple sclerosis, diabetes, or arthritis.

The synthesis of 2-aminocyclohexane carboxylic acids was performed according to the procedure developed by Davies *et al.*,[15] which stereoselectively provides either the *cis* or the *trans* isomer. The amino acids were Fmoc protected using 9-fluorenylmethyl succinimidyl carbonate according to Milton *et al.*[16] to give Fmoc-cACHC-OH and Fmoc-tACHC-OH, respectively.



A strong preference was imposed on the synthesis of cyclic penta- and hexapeptides, as they are known to be conformationally homogeneous. Larger cyclic peptides would presumably yield a higher variety of conformations, smaller peptides would not be able to present a sufficient part of the recognition sequence and would also not undergo facile cyclization. Noteworthy, the peptides do not contain any other amino acid with conformational bias, except the 2-aminocyclohexane carboxylic acid. Hence, it should be possible to identify basic and general principles regarding the influence of ACHC on the conformation of cyclic penta- and hexapeptides. The peptides given in Table 1 were synthesized containing either *cis*- or *trans*-2-aminocyclohexane carboxylic acid (cACHC and tACHC, respectively).

The linear precursors of the peptides with the sequence c-(-Ile-Asp-Ser-ACHC-Leu-Asn-) and c-(-Asp-Ser-ACHC-Leu-Asn-), respectively, are obtained by solid phase peptide synthesis on 2-chlorotrityl resin according to the Fmoc/tBu protection scheme. The peptides were cyclized in solution under pseudo high dilution conditions using a dual syringe pump to avoid dimerisation.[17] After deprotection of the permanent protecting groups the peptides were purified by RP-HPLC and characterized using MALDI-ToF MS and NMR techniques.

For all peptides a series of NMR experiments was performed in D_6–DMSO to obtain information on conformationally relevant parameters such as proton–proton distances using ROE (rotating frame Overhauser effect),[18] coupling constants, and temperature gradients of the NH chemical shift to identify possible intramolecular hydrogen bonds. The proton–proton distances were used as constraints for the generation of an initial structure for molecular dynamics calculations. Torsion angle variation (φ with 60° increment) resulted in 3600 (pentapeptides) or 16800 conformers (hexapeptides), respectively. The lowest energy conformations were used as starting structures for a restrained molecular dynamics simulation in a cubic solvent box containing approximately 300 DMSO molecules (200 ps). The resulting system was examined in a 1.2 ns free molecular dynamics calculation. The trajectory was clustered and resulted in one or two structural proposals.

Two conformations with a ratio of 2 : 3 were obtained for **P1** according to the simulation. Presumably due to fast exchange between the two conformers only one set of NMR signals was obtained. Both conformations satisfy the determined ROE distances within an error margin of 10%. The less populated structure **P1(a)** presents the cACHC in the central position of a pseudo-γ-turn (Ψγ-turn). This is a γ-turn, which is expanded by one carbon atom (C_8-conformer). It is not stabilized by a hydrogen bond, as the amide proton of Leu points to another side of the molecule than the CO group of Ser. This is supported by the relatively high absolute value of the Leu NH temperature gradient $\Delta\delta/\Delta T$ (-4.4 ppb K^{-1}). Correspondingly, a complementary βI-turn around Leu-Asn-Asp-Ser is present (Fig. 1) as shown by a strong ROE between Asp NH and Ser NH and a medium ROE between Asn NH and Asp NH. This conformation is stabilized by one hydrogen bond from serine NH to leucine CO and another one from cACHC NH to the oxygen in the side chain of serine. Both hydrogen bonds are verified by the temperature gradients of the NH shift value (-0.5 ppb K^{-1} for cACHC NH and -1.9 ppb K^{-1} for Ser NH).

The higher populated conformation **P1(b)** contains cACHC in position i of a β-turn-like structure. A C_{10} conformation (10 atoms in the "ring" formed by the hydrogen bond cACHC CO ⟵ Asp NH) is found like in usual β-turns, but the torsion angles of the amino acids found in the position $i + 1/i + 2$ do not match the values of classical β-turn structures. A strong NOE can be observed between Asp NH ($i + 2$) and Asn NH ($i + 3$) which is typical for most types of β-turns. A hydrogen bond is obviously not being formed, as shown by the relatively high absolute value of the temperature gradient of the Asp NH shift value (-3.8 ppb K^{-1}). Simultaneously, cACHC occupies position $i + 2$ of the γ-turn Asp-Ser-cACHC, which is stabilized by a hydrogen bond from cACHC NH to Asp CO. The absolute temperature gradient value of the cACHC NH shift value is very small as discussed above.

An analogous conformation with the β-amino acid in the i position of a β-turn is found for the solution structure of the hexapeptide **P3** which also contans cACHC. A βI-turn stabilized by a hydrogen bond (ACHC CO ⟵ Ile NH) is proven by the temperature gradient of the chemical shift of Ile NH (-0.05 ppb K^{-1}). This finding is supported by a medium ROE between Leu NH and Asn NH. The expected strong ROE between Ile NH and Asn NH cannot be located due to signal overlap.

In peptide **P2** (Fig. 2), where *trans*-configured ACHC is incorporated, no predominant secondary structural element can be located. The conformational analysis shows that all carboxylic groups are directed towards one side of the molecule and all amide protons point to the other side. This can be explained by the structure of the β-amino acid: the substituents of the cyclohexane chair are directed towards the front and the back of the molecule, respectively. The rest of the cyclic peptide does not seem to be flexible enough to compensate this restriction.

In the peptide **P4** (Fig. 3) the ring apparently is big enough to allow for such compensation and tACHC occupies position $i + 1$ of a β-turn-like structure (C_{11} conformer, Ψβ-turn) with the sequence Ser-tACHC-Leu-Asn. This turn is stabilized by a strong hydrogen bond Ser CO ⟵ Asn NH as proven by the positive value of $\Delta\delta/\Delta T$ ($+0.6$ ppb K^{-1}). Obviously, *trans* configured 2-aminocyclohexane carboxylic acid in the eq/eq conformer with a μ angle of 60° is compatible with the steric requirements of position $i + 1$ of a β-turn in a cyclic hexapeptide.

The peptides were designed as potential models for the binding loop of VCAM-1 located on a surface exposed loop of VCAM-1 (the CD-loop). The hitherto unknown conformational preferences of 2-aminocycloalkane carboxylic acids should be investigated and this knowledge subsequently be utilized for the future design of peptide antagonists of protein–protein interactions. In the postulated recognition sequence of VCAM-1, two turns are found according to the X-ray

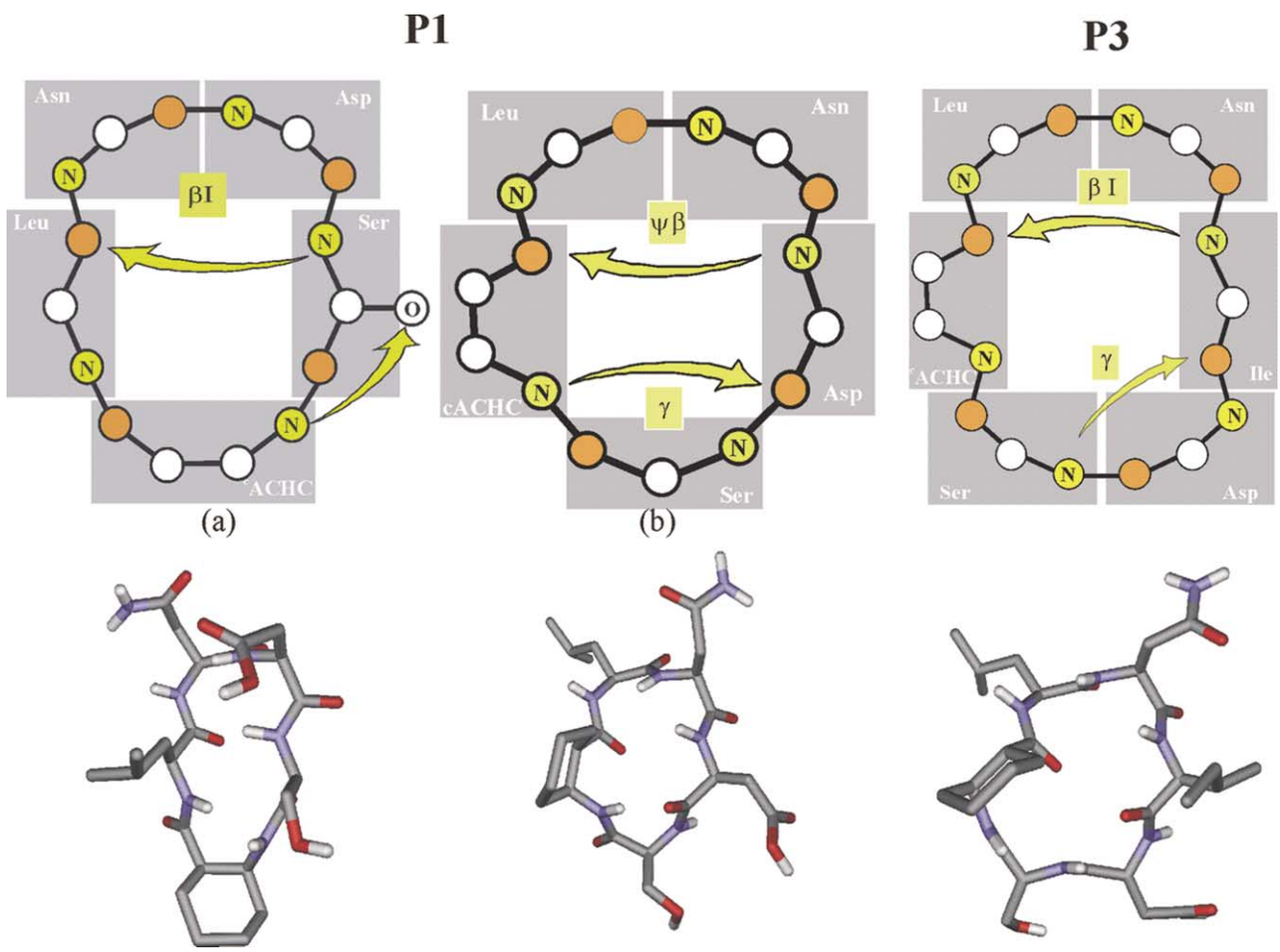


Fig. 1 Conformations of **P1** (a) minor conformer, (b) major conformer and **P3**.

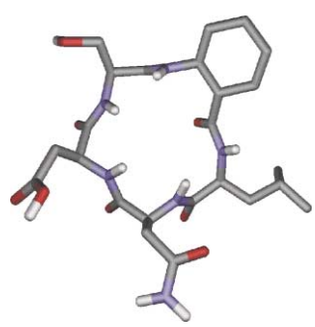


Fig. 2 Conformation of **P2** c-(-Asp-tACHC-Leu-Asn-).

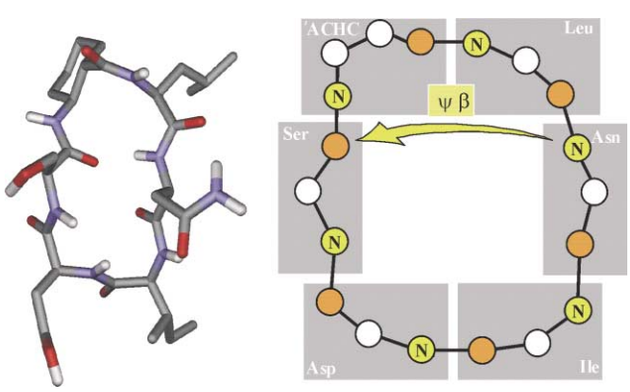


Fig. 3 Conformation of **P4** c-(-Ile-Asp-tACHC-Leu-Asn-Asp-).

structure:[19] a βI-turn at $T^{37}Q^{38}I^{39}D^{40}$ and a βVIa-turn-like conformation at $S^{41}P^{42}L^{43}N^{44}$ with a *trans*-peptide bond between Pro and Leu, which causes an S-type geometry.

An overlay of the synthetic peptides with the putative recognition sequence shows only minimal accordance especially for the side chains of Ile, Asp, and Ser. Table 2 gives the RMS values of the overlay of the peptides with the backbone TQIDSPLN epitope (amino acids Ile (for **P3** and **P4**), Asp, and Ser; overlay of N, C^{α}, C^{β}, C, O).[20] The RMS values increase significantly, when the C^{β} positions are included. Moreover, the cyclic peptides contain only the truncated sequence in a conformationally constrained manner. This leads to the assumption that no imitation of VCAM-1 is possible, as especially the side chains (vectors $C^{\alpha} \longrightarrow C^{\beta}$) are necessary for recognition and binding.

The overlay of all peptides with the recognition sequence is shown in Fig. 4. It can be clearly seen that the side chains of Ser and Ile and the peptide bonds point to different directions. The vectors $C^{\alpha} \longrightarrow C^{\beta}$ of the amino acids Ile, Asp, and Ser, respectively, are directed differently in the peptides and the VCAM-1 loop. Hence, no imitation of the loop structure by the peptides seems to be possible.

Even the assumption of a possible induced fit for the interaction between VCAM-1 and VLA-4 might not be able to accommodate an agreement of the structures because of the obvious large differences between the two structures. These findings are in good accordance with the results of biological tests, which show no inhibition of the adhesion of $\alpha_4\beta_1$-presenting Ramos cells to immobilized VCAM-1 by peptides **P1**–**P4** in the low μM range.[21]

Possible reasons for these results are the different structures or the fact that the binding sequence is not presented in a sufficient manner. Potentially the peptide epitopes are too short to present the minimal recognition sequence. Cyclic octapeptides have been synthesized by Quan *et al.*[22] and were tested as possible inhibitors with low μM affinity.

Table 2 RMS values for the overlay of **P1** to **P4** respectively, with the IDSPLN epitope of VCAM-1

Peptide	RMS value (N, C^{α}, C(O), O)/Å	RMS value (N, C^{α}, C^{β}, C(O), O)/Å
P1 minor conformation	0.586	0.936
P1 major conformation	1.266	1.546
P2	0.943	1.231
P3	1.112	1.493
P4	1.352	1.565

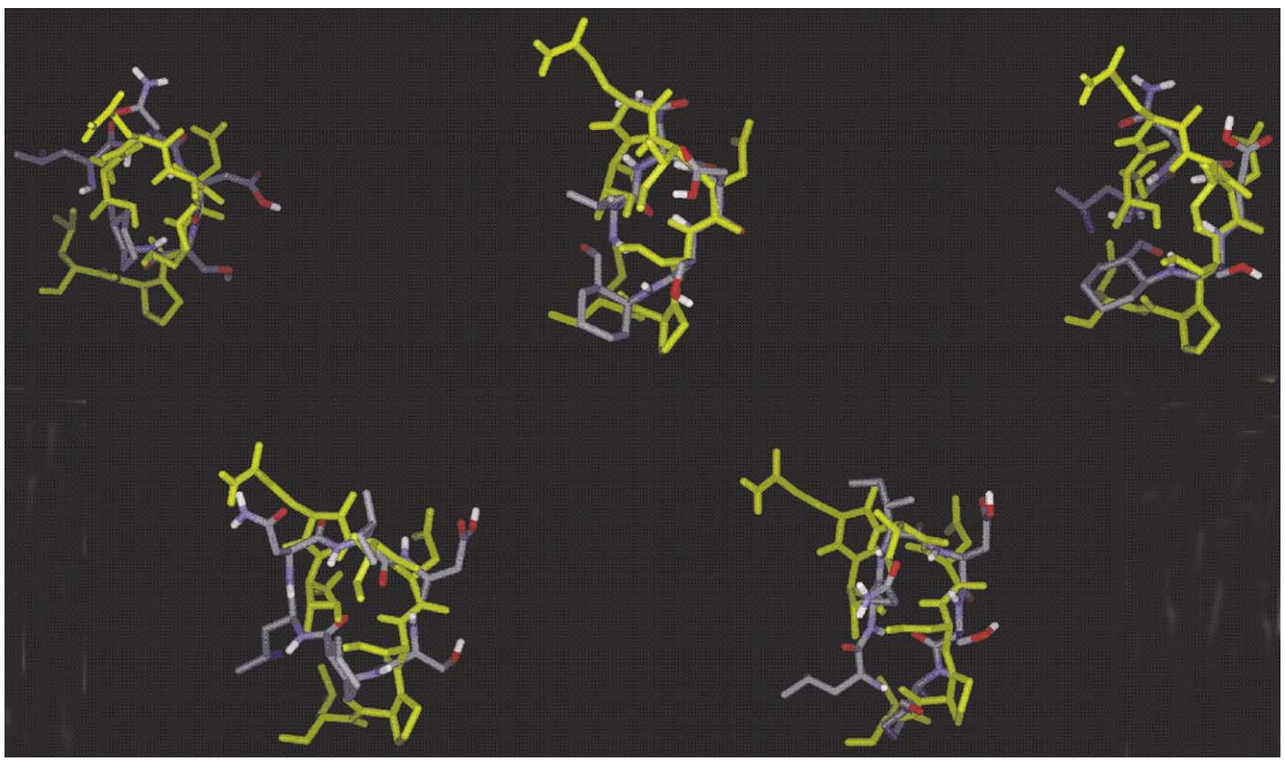


Fig. 4 Overlay of the CD-loop of VCAM-1 [18] (yellow) and peptides **P1** (a), (b), **P2**, **P3**, **P4**.

In several tetra- and pentapeptides β-homoaminoacid residues preferably occupy the central position of a modified γ-turn (Ψγ-turn) and even override the preferences of other elements such as D-amino acids.[3] Consequently, there is evidence that β-homoaminoacids can act as γ-turn mimetics. In the case of 2-aminocycloclohexane carboxylic acids no clear preference for γ-turn induction can be found, especially in the case of hexapeptides. The resulting structures lead to the assumption that β-amino acids, especially 2-aminocyclohexane carboxylic acids, occupy Ψγ-turns preferentially in cyclic tetra- or pentapeptides. In larger peptides they may also be found in pseudo-β-turns. Only the minor conformation of **P1** contains the β-amino acid in the central position of a Ψγ-turn. In the other cases the 2-aminocyclohexane carboxylic acids participate in the formation of β-turns. cACHC is predominantly found in the position i of different β-turns. Peptide **P2** containing *trans*-ACHC displays no defined hydrogen bonded turn structure, which may be ascribed by the ring size and the steric restrictions of the amino acid. Interestingly, this β-amino acid acts as a strong inducer of helical structures in linear peptides.[4] In **P4** tACHC can be found in the position $i + 1$ of a Ψβ-turn, forming a C_{11}-conformer. For all peptides new secondary structures with so far unknown locations of the β-amino acid have been elucidated. Further investigations in order to reveal more details on the role of 2-aminocyclohexane carboxylic acids as proline mimetics in cyclic peptides and to exploit these findings for the rational design *e.g.* of mimetics of the VCAM-1 binding loop are currently in progress.

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