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Further Evidence for 2-Alkyl-2-carboxyazetidines as γ-Turn Inducers

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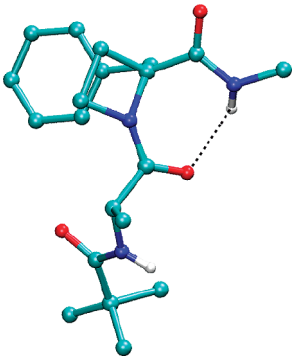

Reverse turns, a common motif in proteins and peptides, have attracted attention due to their relevance in a wide variety of biological processes. In an attempt to artificially imitate and stabilize these turns in short peptides, we have developed versatile synthetic methodologies for the preparation of 2-alkyl-2-carboxyazetidines and incorporated them into the $i+1$ position of model tetrapeptides, where they have shown a tendency to induce γ-turns. However, to ascertain the general utility of these restricted amino acids as γ-type reverse turn inducers, it was then required to study the conformational preferences when located at other positions. To this end, model tetrapeptides R-CO-Ala-Xaa-NHMe, containing differently substituted azetidine moieties (Xaa = Aze, 2-MeAze, 2-BnAze) at the $i+2$ position, were synthesized and subjected to a thorough conformational analysis. The theoretical and experimental results obtained, including the X-ray diffraction structure of a dipeptide derivative containing this skeleton, provide evidence that the 2-alkyl-2-carboxyazetidine scaffold is able to efficiently induce γ-turns when incorporated into these short peptides, irrespective of their localization in the peptide chain.

Introduction

Reverse turns, non-repetitive peptide secondary structures, have attracted attention as they are frequently localized in the exposed surface of peptides and proteins and have therefore proven to be relevant in biomolecular recognition events. In addition, they have the advantage of being very simple structurally, which facilitates their simulation by means of small organic molecules.[1−4] Different efforts have been dedicated to the search of scaffolds able to mimic or to induce reverse turns, which could be of potential utility in the modulation of peptide−protein and protein−protein interactions.[5−7] In the latter years, the modulation of these interactions has deserved much interest due to their importance in a great number of cellular processes. In addition, there is evidence to suggest that misregulation of these

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interaction is implicated in the development and maintenance of pathological processes.[8−10]

There are several types of reverse turns, with the β-turn being the most frequent non-repetitive motif in proteins.[11,12] A β-turn is a non-helical region of the protein, involving four consecutive residues, where the polypeptide folds back into itself, with a distance between the α-carbons of the first and fourth residues ($\alpha C^{i}-\alpha C^{i+3}$) smaller than 7 Å.[13,14] The second most common reverse turn is the γ-turn that consists of three consecutive amino acids structured in a seven-membered pseudocycle.[14−17] Frequently, these reverse turns are stabilized by an intramolecular H-bond between the backbone CO of the first residue (*i*) and the backbone NH of the last one [the fourth ($i + 3$) in β-turns, and the third ($i + 2$) in γ-turns].[17,18]

An adequate inducer of these turns not only should be able to induce the turn topography in the peptides where it is incorporated but also must fulfill a series of requirements. Thus, it should be able to incorporate the side chain of the corresponding amino acids, as side chains are important in recognition events, and should have N- and C-terminal groups suitable for its insertion into larger peptides. While the number of β-turn mimetics is quite appreciable[5,6,19] and some of them fulfill the indicated requirements, the organic structures able to imitate or to induce γ-turns are more scarce.[4,20−24] Among the latter, we have recently developed a synthetic methodology to obtain 2-alkyl-2-carboxyazetidines, which are able to induce γ-turns when incorporated at the $i + 1$ position of simplified tetrapeptide models.[24] In contrast, previous studies based on NOESY spectra of peptides containing the natural amino acid azetidine-2-carboxylic acid (Aze) have pointed out its propensity for β-bend formation.[25−27] On the other hand, the higher homologue proline tends to induce β-turns, mainly of type I and II, when placed at the $i + 1$ position of the turn.[24,28,29] Recent studies on *N*-acyldipeptide *N*-alkylamides containing the conformationally constrained 2-MePro have shown a higher tendency of this residue to promote β-turns than Pro when located at the $i + 1$ position, with a propensity to helical type III β-turns.[30,31] However, the incorporation of Pro derivatives at the $i + 2$ position of tetrapeptides normally leads to the induction of type VI β-turns,[29] whereas 2-MePro at this position seems to be unable to nucleate β-turn conformations in homochiral peptides.[31] In order to ascertain if there is a change in the conformation induced by 2-alkyl-azetidine derivatives depending on their situation at the peptide chain, as in the case of proline analogues, we decided to synthesize and study the conformational behavior of model tetrapeptides R-CO-Ala-Xaa-NHMe (Xaa = Aze, 2-MeAze, 2-BnAze), with the azetidine moiety at the $i + 2$ position. For comparative purposes, we have also prepared and analyzed the corresponding peptides that incorporate Pro or 2-MePro residues at this position.

Results and Discussion

Synthesis. H-2-MePro-OMe[32] (**1b**) and H-Aze-OMe (**2a**) were prepared from H-2-MePro-OH and *tert*-butyloxycarbonyl (Boc) *N*-protected Aze (Boc-Aze-OH), respectively, by treatment with thionyl chloride in MeOH. On the other hand, H-2-MeAze-OMe [(***R*,*S*)-2b**] was synthesized by catalytic hydrogenation of the *p*-(methoxy)benzyl (Pmb) derivative Pmb-2-MeAze-OMe,[24] using $Pd(OH)_2$ as catalyst, and H-2-BnAze-OMe [(***R*,*S*)-2c**] was prepared as previously described.[33a] It is interesting to note that the azetidine derivative H-2-BnAze-OMe (**2c**) was obtained as a non-racemic enantiomeric mixture [Pmb-2(*S*)-BnAze-OMe:Pmb-2(*R*)-BnAze-OMe = 3.5:1]. This was due to the stereoselective construction of the precursor azetidinone ring, from chiral H-Phe-OMe, since the cyclization reaction of β-ramified amino acids proceeds by way of a planar enolate intermediate, which possess axial chirality, according to the concept of memory of chirality.[33b,c] However, this memory process was not observed for β-unbranched amino acids, such as Ala.[33b,c]

The synthesis of dipeptide derivatives R-CO-Ala-Xaa-NHMe, which incorporate the conformationally restricted amino acid at position $i + 2$, was performed following standard solution peptide coupling procedures (Scheme 1).

Dipeptides **3** and **4** were prepared in good yield (65−85%) by peptide coupling between proline **1a,b** or azetidine **2a**−**c**

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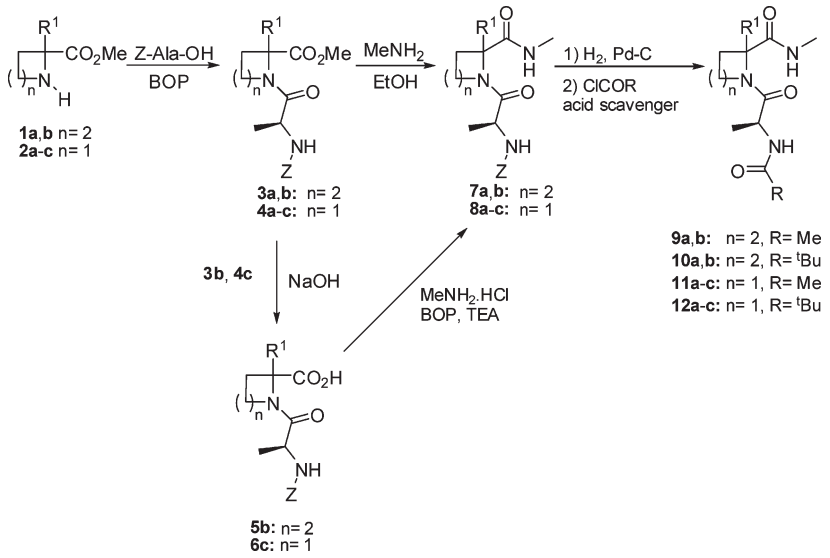
SCHEME 1[a,b]



[a]**a**: R^1 = H. **b**: R^1 = CH_3. **c**: R^1 = CH_2Ph. [b]Stereochemistry on C-2 is (*S*), except for **4b**,**c**, **8b**, **11b**, and **12b**, which are (*R*,*S*)

derivatives and benzyloxycarbonyl (Z) *N*-protected Ala (Z-Ala-OH), using benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) as coupling reagent.[34] Due to the lack of selectivity in the formation of 2(*R*,*S*)-MeAze (**2b**), dipeptide **4b** was obtained as an inseparable 1.1:1 (*S*,*S*):(*S*,*R*) diastereoisomeric mixture. The pair of diastereoisomers of **4c** could be chromatographically isolated from the corresponding 2.2:1 (*S*,*S*):(*S*,*R*) mixture. The diastereoisomeric ratio of **4c** is indicative of the existence of some kinetic resolution during the synthesis of **4c**, since the starting azetidine 2-BnAze [(***R*,*S*)-2c**] has an *S*:*R* enantiomeric ratio of 3.5:1.[33] Next, the C-terminal methyl ester of dipeptides **3** and **4** was transformed into the corresponding methylamides by treatment with a solution of methylamine in MeOH. Following this procedure, compounds **7a** and **8a,b** were obtained in excellent yield (95−97%), although this synthetic route did not allow the preparation of Z-Ala-2-MePro-NHMe (**7b**) and Z-Ala-2(*S*)-BnAze-NHMe (**8c**). To overcome this problem, an alternative synthetic route was explored starting from carboxylic acids **5b** and **6c**, which were obtained by saponification of dipeptides **3b** and **4c**, respectively. The subsequent coupling of acids **5b** and **6c** and $MeNH_2 \cdot HCl$ in the presence of BOP and triethylamine (TEA) afforded **7b** and **8c** in 80% and 59% yield, respectively. The chromatographic isolation of the couple of diastereoisomers **8b** was not feasible; however, the presence of a 1.2:1 (*S*,*S*):(*S*,*R*) mixture was deduced from a HPLC analytical separation using a chiral column, in which two well-differentiated signals were observed.

The benzyloxycarbonyl group of dipeptides **7** and **8** was hydrogenolyzed using Pd−C as catalyst. Subsequently, the free NH_2 was acylated with acetyl or pivaloyl chloride in the presence of TEA and under Ar atmosphere, leading to derivatives **9**−**12** in variable yields, depending both on the chloride derivative and on the starting dipeptide. In general, reactions with pivaloyl chloride led to final compounds in yields around 65−72%, except for dipeptide Piv-Ala-Aze-NHMe **12a** (10%). On the other hand, acylation with acetyl chloride showed a greater difference, with high yield for derivative **11a** (80%) and very low yield for Ac-Ala-2-MePro-NHMe **9b** (7%). With the aim of increasing the efficacy in the formation of acetyl derivatives, compounds **9a**, **11b**, and **11c** were prepared using an alternative method based on the employment of propylene oxide as acid scavenger instead of TEA. Under these conditions, the yield of the acetylation varied from moderate to high (52−88%). Different attempts to isolate the couple of diastereoisomers **11b** and **12b** by chromatography were unsuccessful, but the use of a chiral HPLC analytical column allowed the visualization of both diastereiosomers from the quasi-equimolar mixture of Piv-Ala-2(*R*,*S*)-MeAze-NHMe (**12b**).

Conformational Studies. Theoretical Calculations. Molecular Modeling studies were performed to initially evaluate the ability of 2-alkyl-2-carboxyazetidine derivatives to induce reverse turns when incorporated at the $i+2$ position of peptides. These studies were carried out on simplified tetrapeptide models, Ac-Ala-Xaa-NHMe [Xaa = Pro (**9a**), 2-MePro (**9b**), Aze (**11a**), and 2(*S*)-MeAze (**11b**)], in which an acetyl and an *N*-methylcarboxamide group were used as a simplification of the N- and C-terminal amino acid residues, respectively. Conformational studies on these dipeptides were carried out using AMBER as the force field, implemented in InsightII (version 2000.1, Biosym Tech, San Diego, CA), and different initial conformations of each peptide. The different conformers obtained within a +3 kcal mol^{-1} window from the global minimum were clustered into families according to the torsion angles of the peptide skeleton (ϕ and ψ).

To analyze the possible existence of *cis*/*trans* isomers around the tertiary amide bond, the dihedral angle ω of this bond was measured. It is worth noticing that for dipeptides that include a residue of Pro or Aze, the global minimum corresponds to a *cis* disposition of the Ala-Xaa amide bond, whereas for derivatives that incorporate an α-methyl amino acid [2-MePro or 2(*S*)-MeAze], the global minimum has a *trans* disposition. This might be due to the

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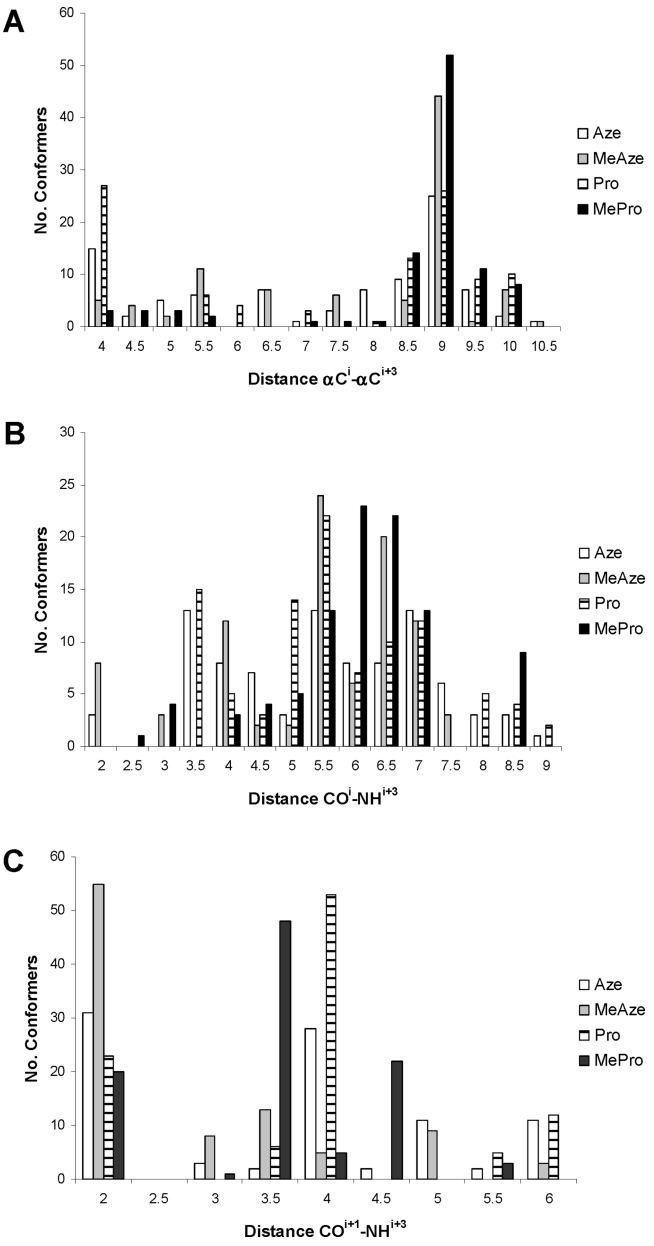

FIGURE 1. Distance distribution (Å) for Ac-Ala-Xaa-NHMe conformers (Xaa as indicated) within 3 kcal mol^{-1} window from the global minimum. (A) $\alpha C^{i}-\alpha C^{i+3}$ distance. (B) $CO^{i}-NH^{i+3}$ distance. (C) $CO^{i+1}-NH^{i+3}$ distance.

presence of the methyl group at the α position of the pyrrolidine or azetidine ring, which increases the population of the *trans* isomer, because of steric hindrance between this methyl group and the side chain of the preceding amino acid in the *cis* rotamer, as previously found for simple 2-MePro derivatives.[35,36]

The minimum energy conformers (3 kcal mol^{-1} from the global minimum) were analyzed to study the possible existence of reverse turns. In this sense, the distance between the C-α carbon of the first and fourth residue ($\alpha C^{i}-\alpha C^{i+3} \leq 7$ Å)

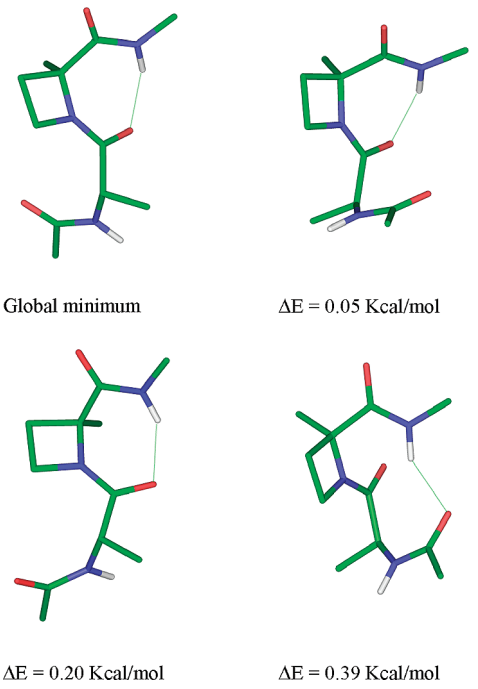

FIGURE 2. Representative minimum energy conformers of Ac-Ala-2(*S*)-MeAze-NHMe (**11b**).

and the distance between the oxygen of the carbonyl group of the first residue and the amide proton of the fourth ($CO^{i}-NH^{i+3} \leq 2.5$ Å) were measured to evaluate the existence of β-turns (Figure 1, A and B). In parallel, to assess the existence of γ-turns, the distance between the CO of the second residue and the amide proton of the fourth ($CO^{i+1}-NH^{i+3} \leq 2.5$ Å) was measured (Figure 1, C). These data showed that around 40% of the minimum energy conformers of Ac-Ala-Pro-NHMe have distances between the α carbons of the first and fourth residues within those expected for β-turn secondary structures, but the $CO^{i}-NH^{i+3}$ distance is greater than 2.5 Å, indicating the lack of the characteristic H-bond and the adoption of open β-turn-like conformations. In these conformers the Ala-Pro peptide bond was always *cis*, which is indicative of a type VI β-turn.[29] On the other hand, the percentage of conformers that fulfils the type VI β-turn requirements decreased approximately up to 30% for peptides incorporating Aze or 2-MeAze and even more (up to 10%) for the Ac-Ala-2-MePro-NHMe derivative (**9b**). Additionally, the molecular modeling studies indicated the presence of conformers with the characteristic H-bond of γ-turns, with the lower population for derivatives containing Pro or 2-MePro (around 20%) and increased percentages for those with a residue of Aze (30%) and specially of 2-MeAze (over 50%). Finally, the global minima of the dipeptide derivatives that contain Pro, 2-MePro or Aze did not adopt a reverse turn structure, whereas for Ac-Ala-2(*S*)-MeAze-NHMe this minimum, as well as other low energy minima, corresponded to γ-turn conformations (Figure 2).

On the whole, our molecular modeling studies suggest that the 2-alkyl-2-carboxyazetidines in position $i + 2$ of model tetrapeptides are able to stabilize γ-turn conformations, although these theoretical data need verification from experimental studies.

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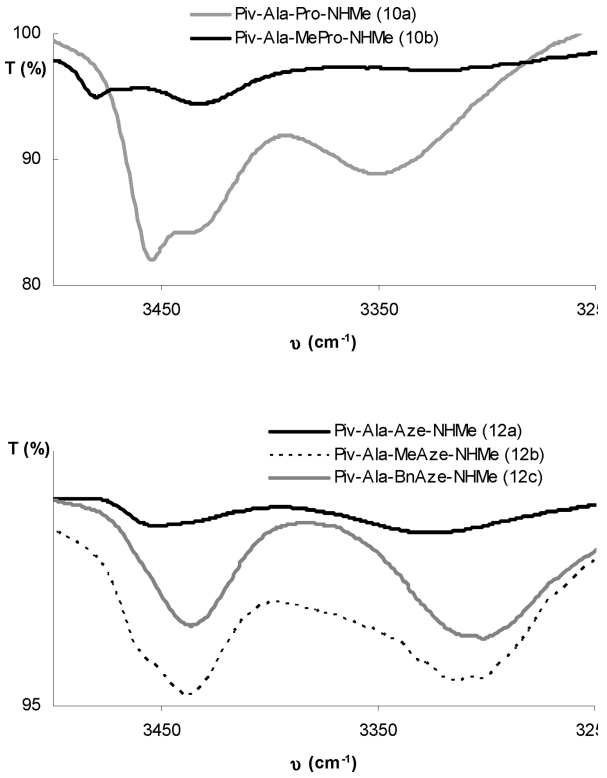

FIGURE 3. NH stretch region of FT-IR absorption spectra of dipeptide derivatives Piv-Ala-Xaa-NHMe (**10a,b** and **12a**−**c**).

Conformational Studies in Solution (IR and ^{1}H NMR). FT-IR absorption studies in $CDCl_3$ were performed to analyze the NH stretching bands (amide A) that absorb in the 3460−3410 and 3380−3300 cm^{-1} regions, corresponding to free and H-bond NH moieties, respectively.[37−41] With the exception of the dipeptide that incorporates Pro, the shape and frequency of the NH bands did not vary with the concentration (between 2 and 20 mM). The FT-IR absorption spectrum of the dipeptide derivatives **7**−**12** showed, in general, an equilibrium between free and intramolecular hydrogen-bonded states, with a decrease in the intensity of the associated NH band when the N-terminal moiety is a benzyloxycarbonyl group.

As an example, Figure 3 shows the FT-IR absorption spectra of dipeptide derivatives with a pivaloyl group at the N-terminus. These data indicate that for dipeptides containing Pro or 2-MePro residues, the free NH band is more intense than the associated NH band, whereas compounds that incorporate azetidine-2-carboxylate derivatives show similar intensity for both bands. This suggests that peptides with an azetidine residue at the $i + 2$ position are able to form intramolecular H-bonds more easily than those analogues bearing Pro.

The conformational study of derivatives **7**−**12** in solution was completed by ^{1}H NMR analysis. Some of these derivatives show homologous pairs of signals in their ^{1}H NMR spectra due to the *cis*/*trans* isomerism around the Ala-Xaa amide bond. On the other hand, the duplicity of signals in the ^{1}H NMR spectra of dipeptides **8b**, **11b**, and **12b** was attributed to the presence of two diastereoisomers in each case, since their intensities neither vary with the change of solvent nor coalesce in temperature studies. The assignment of the *cis*/*trans* conformations of the Ala-Xaa amide bond was done following the criteria previously reported for peptides incorporating Pro residues (see Supporting Information for details).[2,39,42,43] It has been observed that the population of the *cis* conformers increases moderately with the polarity of the solvent. Thus, for Pro or Aze derivatives (R^1 = H) a 13−28% population of *cis* rotamers were observed in $CDCl_3$ versus 19−37% values in DMSO. Moreover, there is a quasi total stabilization of the *trans*-Ala-Xaa peptide bond when R^1 is an alkyl substituent (Me or Bn). In fact, only MeAze derivatives **8b**, **11b** and **12b** showed presence of *cis* rotamers in DMSO (below 14%).

To evaluate the ability of Aze and Pro residues at the $i + 2$ position of model tetrapeptides to induce reverse turns, the possible existence of H-bonds characteristic of these turns was analyzed through the chemical shifts and the temperature coefficients of the amide NH protons.[2,37,44] In this sense, it is assumed that in DMSO temperature coefficients equal or less than 3 ppb/K (in absolute value) are indicative of solvent-shielded NH, suggesting intramolecular hydrogen bond in small peptides. On the contrary, values higher than 4 ppb/K implies that the NH is accessible to the solvent, whereas those between 3 and 4 ppb/K are not conclusive. Additionally, chemical shifts of the amide NH protons above 7 ppm in $CDCl_3$ together with small variations when the solvent is changed to DMSO are indicative of the participation of these amide proton in an intramolecular hydrogen bond.[37] It was observed that, with the sole exception of derivative **11c**, the chemical shift of the Ala NH in these dipeptide derivatives is lower than 7 ppm (Table 1, *trans* rotamers). These data together with the variation of δ upon solvent change from $CDCl_3$ to DMSO (over 0.88 ppm) and the temperature coefficients above 5 ppb/K in absolute value, are indicative of solvent accessibility of this NH in both *trans* and *cis* (data not shown) isomers. On the other hand, the behavior of the amide proton of the NH-Me moiety depends on the conformation of the Ala-Xaa peptide bond and on the nature of the Xaa amino acid that was incorporated into the dipeptide. Thus, for peptides with a *trans* disposition of the amide bond preceding Pro and 2-MePro residues (**7a,b**, **9a,b**, and **10a,b**), the chemical shift of this NH in $CDCl_3$ and the values of the temperature coefficients above 4 ppb/K (in absolute value) also suggest solvent accessibility. On the contrary, the *trans* rotamers of azetidine-containing dipeptides have chemical shift values for the NH-Me amide proton above 7 ppm in $CDCl_3$. An analysis of the variation of δ upon solvent change to DMSO-d_6 shows two well-differentiated groups. First, derivatives with an Aze residue (**8a**, **11a**, **12a**) showed a deviation of $\Delta\delta$ from $CDCl_3$ to DMSO of around 0.5 ppm,

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TABLE 1. Chemical Shifts and Temperature Coefficients for the NH Amide Protons of *Trans* Isomers of Compounds 7−12

		δNH-Ala (ppm)		δNH-Me (ppm)		$\Delta\delta/\Delta T$[a]	
compound		$CDCl_3$	DMSO	$CDCl_3$	DMSO	NH-Ala	NH-Me
Z-Ala-Pro-NHMe	**7a**	5.65	7.50	6.74	7.69	−6.8	−4.4
Ac-Ala-Pro-NHMe	**9a**	6.37	8.11	6.61	7.69	−5.3	−4.4
Piv-Ala-Pro-NHMe	**10a**	6.52	7.46	6.62	7.70	−5.0	−4.3
Z-Ala-2-MePro-NHMe	**7b**	5.59	7.47	6.79	7.32	−7.0	−4.3
Ac-Ala-2-MePro-NHMe	**9b**	6.49	8.08	6.54	7.31	−6.5	−4.3
Piv-Ala-2-MePro-NHMe	**10b**	6.54	7.42	6.68	7.28	−6.0	−4.2
Z-Ala-2(*S*)-Aze-NHMe	**8a**	5.41	7.57	7.32	7.81	−6.2	−4.7
Ac-Ala-2(*S*)-Aze-NHMe	**11a**	6.30	8.17	7.31	7.86	−5.8	−4.4
Piv-Ala-2(*S*)-Aze-NHMe	**12a**	6.22	7.50	7.29	7.84	−5.3	−4.0
Z-Ala-2(*R*,*S*)-MeAze-NHMe	(***S***)-**8b**[b]	5.42	7.59	7.87	7.75	−6.8	−2.9
	(***R***)-**8b**[b]	5.42	7.66	7.93	7.66	−6.5	−1.7
Ac-Ala-2(*R*,*S*)-MeAze-NHMe	(***S***)-**11b**[b]	6.42	8.16	7.83	7.78	−5.3	−2.3
	(***R***)-**11b**[b]	6.48	8.24	7.83	7.78	−5.2	−2.3
Piv-Ala-2(*R*,*S*)-MeAze-NHMe	(***S***)-**12b**[b]	6.32	7.55	7.86	7.82	−5.9	−3.0
	(***R***)-**12b**[b]	6.25	7.73	7.86	7.73	−5.9	−1.6
Z-Ala-2(*S*)-BnAze-NHMe	**8c**	5.44	7.61	8.09	7.98	−7.0	−2.0
Ac-Ala-2(*S*)-BnAze-NHMe	**11c**	7.22	8.18	8.14	8.04	−5.8	−2.1
Piv-Ala-2(*S*)-BnAze-NHMe	**12c**	6.49	7.49	8.16	8.04	−5.8	−2.3

[a]Values in ppb/K. Δδ measured in DMSO-d_6, 30−60 °C. [b]The NMR data correspond to (S) or (R) configuration and they might be interchanged.

whereas those with a residue of 2-alkyl-2-carboxyazetidine (**8b,c**, **11b,c**, **12b,c**) presented smaller variations (0.04−0.27 ppm). This different behavior was also observed in the values of NH-Me temperature coefficients. Dipeptides containing Aze (**8a**, **11a** and **12a**) have values equal or higher than 4 ppb/K (in absolute values), which suggests lack of intramolecular H-bonds, in agreement with the $\Delta\delta$ values upon solvent change. On the contrary, values of $\Delta\delta/\Delta T$ for derivatives containing 2-MeAze and 2-BnAze were lower than 3 ppb/K (in absolute value), indicative of solvent protection of this amide proton, and thus, of the possible existence of an intramolecular H-bond for these compounds. Finally, among isomers with a *cis* disposition of the Ala-Xaa peptide bond, only compound **10a** showed a relatively low value of $\Delta\delta$ for the NH-Me amide proton (0.34 ppm) and a temperature coefficient in the uncertainty range (−3.3 ppb/K), which suggests the possible adoption of a type VI β-turn, in agreement with the molecular modeling studies. On the whole, the NMR conformational studies show that the incorporation of 2-alkyl-2-carboxyazetidines at position $i + 2$ of simplified tetrapeptide models favor the formation of a H-bond involving the NH group of the C-terminal residue. It is worth mentioning that the presence of an R^1 alkyl substituent at the α position of the azetidine ring is an essential requirement for the establishment of this intramolecular H-bond.

Although 2D NOESY correlation studies, both in $CDCl_3$ and in DMSO, were done with the aim of establishing the global 3D structure and thus the carbonyl group involved in the H-bond, no conclusive results were obtained.

Summarizing, the conformational studies in solution carried out by FT-IR and NMR on simplified tetrapeptide models R-CO-Ala-Xaa-NHMe support the existence of reverse turn conformations in those dipeptides incorporating 2-alkyl-2-carboxyazetidine residues. However, the IR and NMR data are insufficient to identify the carbonyl group involved in the H-bond and therefore to define the type of turn present in these dipeptides (β- or γ-turn). Molecular modeling studies have shown the presence of both γ- and β-turn conformations within the low energy conformers, with a higher population for γ-turns. However, because of the known overestimation of γ-turns in molecular modeling studies,[13,45] it would not be reliable to ascertain the 3D structure only on these bases. Thus, we decided to try to determine the 3D structure by X-ray diffraction studies.

X-ray Diffraction. With the aim of generating crystals of derivatives **11c** (Ac-Ala-2(*S*)-BnAze-NHMe) and **12c** (Piv-Ala-2(*S*)-BnAze-NHMe) different crystallization conditions were assayed. However, only for compound **12c** were the crystals obtained adequate for X-ray diffraction. It is interesting to point out that a bibliographic search showed that there are scarce examples of small peptides with γ-turn structure in the crystal state.[21,46−51] Peptide **12c** crystallized from hexane in the monoclinic system showing two **12c** molecules (A and B) and two water molecules in the asymmetric unit. Figure 4 shows the molecular conformation that was determined in the crystal state. In agreement with our previous conformational analysis, the three amide bonds of the molecule adopted a *trans* disposition, and in molecule **12c**A the amide NH-Me proton is involved in a H-bond. In this sense, the distance between the nitrogen atom of the methylamino moiety and the oxygen of the carbonyl group of Ala (2.84 Å) and the value of the angle N−H···O (130.7°) are indicative of the existence of intramolecular hydrogen bond characteristic of a γ-turn. Moreover, ϕ and ψ dihedral angles of the 2-BnAze residue ($\phi = -71.4°$ and $\psi = 52.7°$) are within those expected for a inverse γ-turn. On the other hand molecule **12c**B lacks this H-bond, the distance between the nitrogen atom of the methylamino moiety and the oxygen of the carbonyl group of Ala being of 3.44 Å, and the dihedral angles of the 2-BnAze residue are $\phi = -77.8°$ and

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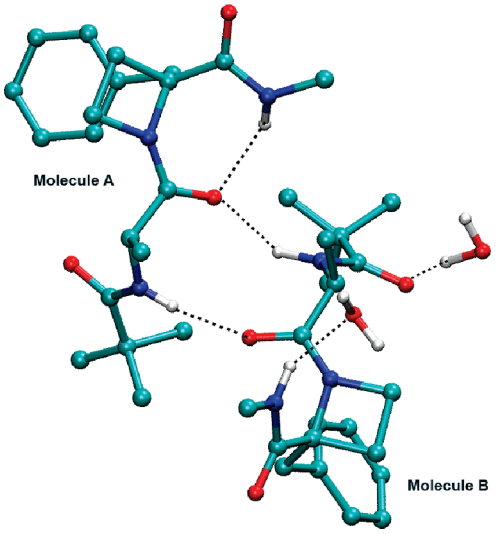

FIGURE 4. Molecular conformation of Piv-Ala-2(*S*)-BnAze-NHMe (**12c**) in the crystal state; hydrogen bonds are indicated by black dotted lines.

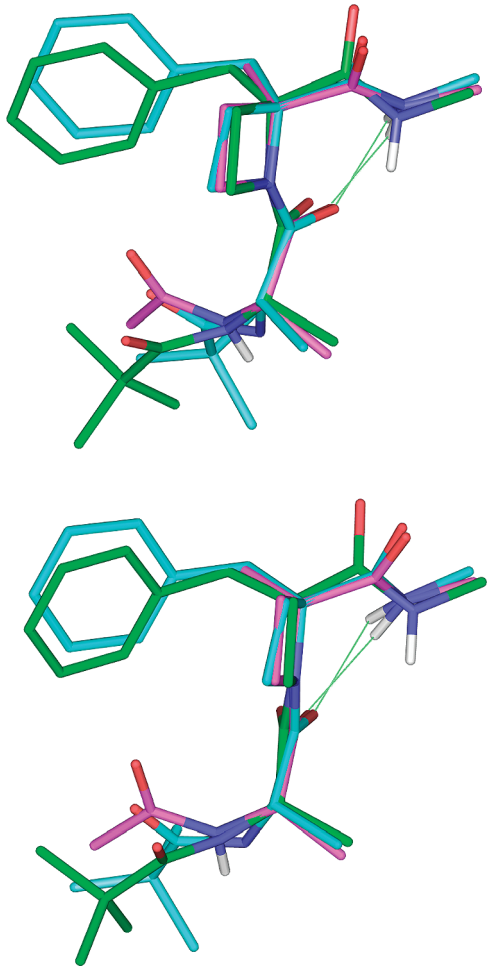

FIGURE 5. Frontal and lateral view of the superposition of the X-ray diffraction structure of Piv-Ala-2(*S*)-BnAze-NHMe **12c** (C atoms in cyan in A molecule and in green in B molecule) and the global minimum conformer of Ac-Ala-2(*S*)-MeAze-NHMe **11b** (C atoms in purple). For clarity, only NH hydrogens are shown.

$\psi = -10.4°$. It is worth pointing out that the overall geometry of molecule B is quite similar to that of the molecule **12c**A, which has the H-bond characteristic of the γ-turn (Figure 5). The lack of this H-bond in molecule B may be due to the presence of water molecules in the crystal structure, as the amide proton of the NH-Me moiety is forming a H-bond with one of the water molecules. On the whole, dipeptide **12c** adopts an overall γ-turn structure in the solid state, corroborating the results obtained in the molecular modeling studies and contributing to the interpretation of IR and NMR data.

Comparison of the X-ray diffraction molecular structures and the global minimum energy conformer found in the molecular modeling studies for Ac-Ala-2(*S*)-MeAze-NHMe (**11b**) showed an excellent agreement between the observed and theoretical 3D structures. As it can be seen in Figure 5, the greater differences are located toward the N-teminal residue, although the dihedral angles of the backbone of Ala residue in the X-ray diffraction structure of **12c** (molecule A $\phi = -89.7°$ and $\psi = 153.9°$, molecule B $\phi = -110.4°$ and $\psi = 133.2°$) are quite similar to those measured in the theoretically generated global minimum of **11b** ($\phi = -120°$ and $\psi = 144°$).

Conclusions

The results of a systematic conformational study by molecular modeling, FT-IR absorption, and ^{1}H NMR of simplified R-CO-Ala-Xaa-NHMe tetrapeptide derivatives incorporating an Xaa residue of 2-alkyl-2-carboxyazetidine at position $i + 2$, together with the X-ray diffraction data of Piv-Ala-2(*S*)-BnAze-Me (**12c**), have indicated the ability of the 2-alkylazetidine moiety to stabilize γ-turn secondary structures in all of the assayed conditions, vacuum, solution, and crystal state. In contrast to Pro derivatives, both in our own studies[24b] as well as in other recently published for Ala-Pro and Ala-2-MePro dipeptides derivatives,[31] and in agreement with our previous analysis of the conformations induced by 2-alkylazetidines at the $i + 1$ position of related peptides,[24] it can be concluded that these restricted amino acids show a tendency to induce γ-turn conformations in short peptides, independently of their location. Besides their interests as small peptide derivatives with γ-turn structure in the crystal state, these 2-alkyl-2-carboxyazetidines are able to support different amino acid side chains and have N- and C-terminal groups adequate for their insertion into peptide chains. Thus, these derivatives could be used as tools to elucidate the bioactive conformation of peptides, allowing a γ-turn scan by sequential replacement of each amino acid residue by the suitably substituted 2-alkyl-2-carboxyazetidine. In addition, they would be interesting skeletons to stabilize γ-turn structural motifs in biological relevant peptides, for which a γ-turn active conformation has or is suspected to have a special relevance. Work in this direction is being done and will be published in due course.

Experimental Section

General Procedure for the Preparation of Dipeptide Derivatives with C-Terminal Amide Groups. Method A. The corresponding dipeptide methyl ester (4.22 mmol) was dissolved in $MeNH_2 \cdot EtOH$ (10 mL, 8 M). After 2 h of reaction the solvent was evaporated to dryness, and when necessary, the residue was

purified by flash chromatography or centrifugal circular thin-layer chromatography, using the system of eluents indicated in each case.

Method B. To a solution of the corresponding dipeptide with the free C-terminal carboxylic acid (0.34 mmol) in CH_2Cl_2 were added $MeNH_2 \cdot HCl$ (3.40 mmol) and BOP (195 mg, 0.44 mmol). The solution was cooled to 0 °C, and TEA (3.84 mmol) was added. After stirring for 6 days at room temperature, the reaction mixture was successively washed with citric acid (10%), $NaHCO_3$ (10%), H_2O, and brine. The organic layer was dried over Na_2SO_4, the solvent was evaporated to dryness, and the residue was purified by flash chromatography using the system of eluents indicated in each case.

Z-Ala-2(*R*,*S*)-MeAze-NHMe (8b). Syrup, 99% yield [from (*R*,*S*)-**4b**, Method A]. Eluent: EtAcO/hexane (8:1). HPLC (Chiralpak, hexane/methanol = 95:5): t_R = 22.9 min (major diastereoisomer), 30.5 min (minor diastereoisomer). Diastereoisomers ratio 1.2:1. ^{1}H NMR ($CDCl_3$): major diastereoisomer δ 7.87 (m, 1H, NH-CH_3), 7.35 (m, 5H, Ph), 5.42 (m, 1H, NH-Ala), 5.10 (s, 2H, CH_2-Z), 4.21 (q, 1H, J = 7.1, α-H, Ala), 4.17 (m, 1H, H-4), 3.97 (m, 1H, H-4), 2.88 (m, 1H, H-3), 2.79 (d, 3H, J = 4.7, CH_3−NH), 2.12 (m, 1H, H-3), 1.72 (s, 3H, 2-CH_3, 2-MeAze), 1.30 (d, 3H, J = 7.1, β-H, Ala); minor diastereoisomer δ 7.93 (m, 1H, NH-CH_3), 7.35 (m, 5H, Ph), 5.42 (m, 1H, NH-Ala), 5.10 (s, 2H, CH_2-Z), 4.17 (m, 1H, H-4), 4.13 (q, 1H, J = 7.0, α-H, Ala), 3.97 (m, 1H, H-4), 2.88 (m, 1H, H-3), 2.80 (d, 3H, J = 4.5, CH_3−NH), 2.12 (m, 1H, H-3), 1.73 (s, 3H, 2-CH_3, 2-MeAze), 1.34 (d, 3H, J = 7.0, β-H, Ala). ^{1}H NMR (DMSO-d_6): major diastereoisomer δ 7.75 (m, 1H, NH-CH_3), 7.59 (d, 1H, J = 7.0, NH-Ala), 7.33 (m, 5H, Ph), 5.00 (s, 2H, CH_2-Z), 4.15 (m, 1H, H-4), 4.00 (m, 1H, H-4), 3.96 (q, 1H, J = 7.0, α-H, Ala), 2.60 (d, 3H, J = 4.3, CH_3−NH), 2.26 (m, 1H, H-3), 2.01 (m, 1H, H-3), 1.52 (s, 3H, 2-CH_3, 2-MeAze), 1.14 (d, 3H, J = 7.0, β-H, Ala); minor diastereoisomer δ 7.67−7.65 (m, 2H, NH-CH_3, NH-Ala), 7.33 (m, 5H, Ph), 5.02 (s, 2H, CH_2-Z), 4.15 (m, 1H, H-4), 4.00 (m, 1H, H-4), 3.96 (q, 1H, J = 7.0, α-H, Ala), 2.57 (d, 3H, J= 4.3, CH_3-NH), 2.26 (m, 1H, H-3), 2.01 (m, 1H, H-3), 1.52 (s, 3H, 2-CH_3, 2-MeAze), 1.15 (d, 3H, J = 7.0, β-H, Ala). ^{13}C NMR ($CDCl_3$): major diastereoisomer δ 174.4 (CO-Ala), 172.6 (2-CO), 155.6 (CO-Z), 136.2 (C-Ph), 128.5, 128.2, 128.0 (CH-Ph), 72.0 (2-C), 66.9 (CH_2-Z), 46.5 (α-C, Ala), 45.8 (4-C), 27.0 (3-C), 26.2 (CH_3-NH), 23.4 (2-CH_3, 2-MeAze), 18.2 (β-C, Ala); minor diastereoisomer δ 174.0 (CO-Ala), 173.4 (2-CO), 155.6 (CO-Z), 136.2 (C-Ph), 128.5, 128.2, 128.0 (CH-Ph), 71.7 (2-C), 66.9 (CH_2-Z), 46.7 (α-C, Ala), 45.7 (4-C), 27.4 (3-C), 26.2 (CH_3-NH), 23.2 (2-CH_3, 2-MeAze), 17.7 (β-C, Ala). ES-MS: 334.0 $[M + 1]^+$, 356.0 $[M + Na]^+$. Anal. Calcd for $C_{17}H_{23}N_3O_4$: C, 61.25; H, 6.95; N, 12.60. Found: C, 61.00; H, 6.71; N, 12.74.

Z-Ala-2(*S*)-BnAze-NHMe (8c). Foam, 59% yield [from (*S*)-**6c**, Method B]. HPLC: t_R = 13.41 min (Novapak, A:B = 30:70). $[\alpha]_D$ = +30.2 (*c* 0.35, $CHCl_3$). ^{1}H NMR ($CDCl_3$): *trans*/*cis* isomers ratio 10:1; *trans* isomer δ 8.09 (c, 1H, J = 4.8, NH-CH_3), 7.33−7.07 (m, 10H, Ph), 5.44 (d, 1H, J = 8.3, NH-Ala), 5.07 (m, 2H, CH_2-Z), 4.04 (m, 1H, α-H, Ala), 3.54 (m, 1H, H-4), 3.50 (d, 1H, J = 13.9, 2-CH_2, 2-BnAze), 3.07 (m, 1H, H-4), 2.87 (d, 1H, J = 13.9, 2-CH_2, 2-BnAze), 2.78 (d, 3H, J = 4.8, CH_3-NH), 2.67 (m, 1H, H-3), 2.15 (m, 1H, H-3), 1.20 (d, 3H, J = 6.9, β-H, Ala). ^{1}H NMR (400 MHz, DMSO-d_6): *trans*/*cis* isomers ratio 10:1; *trans* isomer δ 7.98 (c, 1H, J = 4.5, NH-CH_3), 7.61 (d, 1H, J = 7.6, NH-Ala), 7.36−7.20 (m, 10H, Ph), 5.04 (m, 2H, CH_2-Z), 3.99 (m, 1H, α-H, Ala), 3.76 (m, 1H, H-4), 3.38 (d, 1H, J = 13.9, 2-CH_2, 2-BnAze), 3.21 (m, 1H, H-4), 2.98 (d, 1H, J = 13.9, 2-CH_2, 2-BnAze), 2.67 (d, 3H, J = 4.5, CH_3-NH), 2.24 (m, 1H, H-3), 2.00 (m, 1H, H-3), 1.16 (d, 3H, J = 6.9, β-H, Ala). ^{13}C NMR ($CDCl_3$): *trans* isomer δ 174.1 and 173.8 (CO-Ala and 2-CO), 155.3 (CO-Z), 136.3, 134.6 (C-Ph), 130.3, 128.6, 128.5, 128.2, 128.1, 127.3 (CH-Ph), 75.6 (2-C), 66.9 (CH_2-Z), 46.6 (α-C, Ala), 46.1 (4-C), 39.5 (2-CH_2, 2-BnAze), 26.3 (CH_3-NH), 22.7 (3-C), 18.3 (β-C, Ala). ES-MS: 410.2 $[M + 1]^+$, 432.1 $[M + Na]^+$. Anal. Calcd for $C_{23}H_{27}N_3O_4$: C, 67.46; H, 6.65; N, 10.26. Found: C, 67.21; H, 6.81; N, 10.18.

General Procedure for the *N*-Acylation of Dipeptide Derivatives. Method A. To a solution of the corresponding Z-protected dipeptide (0.73 mmol) in MeOH (15 mL) at 0 °C was added Pd−C (15% w/w). The mixture was hydrogenated at 15 psi and room temperature for 1 h. After filtration of the catalyst, the solvent was evaporated to dryness. The residue was dissolved in dry CH_2Cl_2 (6 mL) and cooled to 0 °C under argon atmosphere. Subsequently, TEA (0.21 mL, 1.53 mmol) and acetyl or pivaloyl chloride (1.17 mmol) were added. After 2 h of reaction at room temperature the reaction was successively washed with citric acid (10%), $NaHCO_3$ (10%), H_2O, and brine. The organic layer was dried over Na_2SO_4, the solvent was evaporated to dryness, and the residue was purified by flash chromatography using the system of eluents indicated in each case.

Method B. Following the same procedure as in Method A, except that, because of the solubility of the final product in H_2O, the aqueous phase was evaporated to dryness and the resulting residue was purified by flash chromatography.

Method C. As in Method A, but using propylene oxide (0.77 mL, 10.92 mmol) instead of TEA. After 15 h of stirring at room temperature the solvent was evaporated to dryness, and the residue was purified by flash chromathography using the system of eluents indicated in each case.

Ac-Ala-Aze-NHMe (11a). Syrup, 80% yield (from **8a**, Method B). Eluent: $MeOH/CH_2Cl_2$ (1:20). $[\alpha]_D$ = −158.1 (*c* 1.13, $CHCl_3$). ^{1}H NMR ($CDCl_3$): *trans*/*cis* isomers ratio 3.5:1; *trans* isomer δ 7.31 (br s, 1H, NH-CH_3), 6.30 (m, 1H, NH-Ala), 4.84 (dd, 1H, J = 9.4, 6.2, H-2), 4.50 (q, 1H, J = 7.0, α-H, Ala), 4.31 (m, 1H, H-4), 4.08 (m, 1H, H-4), 2.80 (d, 3H, J = 4.7, CH_3-NH), 2.70 (m, 1H, H-3), 2.47 (m, 1H, H-3), 1.99 (s, 3H, CH_3-CO), 1.30 (d, 3H, J = 7.0, β-H, Ala); *cis* isomer δ 8.21 (br s, 1H, NH-CH_3), 6.49 (m, 1H, NH-Ala), 4.75 (dd, 1H, J = 10.2, 5.3, H-2), 4.01 (m, 3H, H-4, α-H, Ala), 2.89 (d, 3H, J = 4.7, CH_3-NH), 2.70 (m, 1H, H-3), 2.34 (m, 1H, H-3), 1.99 (s, 3H, CH_3-CO), 1.30 (m, 3H, β-H, Ala). ^{1}H NMR (400 MHz, DMSO-d_6): *trans*/*cis* isomers ratio 2.7:1; *trans* isomer δ 8.17 (d, 1H, J = 6.8, NH-Ala), 7.86 (m, 1H, NH-CH_3), 4.59 (dd, 1H, J = 8.8, 5.9, H-2), 4.32 (q, 1H, J = 6.8, α-H, Ala), 4.27 (m, 1H, H-4), 4.17 (m, 1H, H-4), 2.69 (d, 3H, J = 4.9, CH_3-NH), 2.49 (m, 1H, H-3), 2.16 (m, 1H, H-3), 1.90 (s, 3H, CH_3-CO), 1.24 (d, 3H, J = 6.8, β-H, Ala); *cis* isomer δ 8.37 (m, 1H, NH-CH_3), 8.17 (m, 1H, NH-Ala), 4.85 (dd, 1H, J = 8.8, 3.9, H-2), 4.13 (m, 1H, α-H, Ala), 3.87 (m, 2H, H-4), 2.74 (d, 3H, J = 3.9, CH_3-NH), 2.64 (m, 1H, H-3), 2.16 (m, 1H, H-3), 1.90 (s, 3H, CH_3-CO), 1.24 (m, 3H, β-H, Ala). ^{13}C NMR ($CDCl_3$): *trans* isomer δ 174.5 (CO-Ala), 170.9 (2-CO), 169.7 (CO-CH_3), 61.9 (2-C), 48.8 (4-C), 44.4 (α-C, Ala), 26.1 (CH_3-NH), 22.8 (CH_3-CO), 18.8 (3-C), 17.9 (β-C, Ala); *cis* isomer δ 173.1 (CO-Ala), 171.5 (CO-CH_3), 170.6 (2-CO), 63.0 (2-C), 46.3 (4-C), 46.1 (α-C, Ala), 26.2 (CH_3-NH), 22.3 (CH_3-CO), 22.2 (3-C), 16.1 (β-C, Ala). ES-MS: 228.1 $[M + 1]^+$, 250.1 $[M + Na]^+$. Anal. Calcd for $C_{10}H_{17}N_3O_3$: C, 52.85; H, 7.54; N, 18.49. Found: C, 52.71; H, 7.63; N, 18.29.

Ac-Ala-2(*S*)-BnAze-NHMe (11c). Syrup, 60% yield (from **8c**, Method C). Eluent: acetone/EtAcO (1:2). HPLC: t_R = 3.25 min (Novapak, A:B = 20:80). ^{1}H NMR ($CDCl_3$): δ 8.14 (m, 1H, NH-CH_3), 7.24 (m, 5H, Ph), 7.22 (d, 1H, J = 6.9, NH-Ala), 4.35 (q, 1H, J = 6.9, α-H, Ala), 3.63 (ddd, 1H, J = 9.8, 8.5, 4.9, H-4), 3.56 (d, 1H, J = 13.9, 2-CH_2, 2-BnAze), 3.17 (m, 1H, H-4), 2.97 (d, 1H, J = 13.9, 2-CH_2, 2-BnAze), 2.86 (d, 3H, J = 4.7, CH_3-NH), 2.73 (ddd, 1H, J = 12.2, 9.8, 7.2, H-3), 2.23 (ddd, 1H, J = 12.2, 9.2, 4.9, H-3), 2.05 (s, 3H, CH_3-CO), 1.26 (d, 3H, J = 6.9, β-H, Ala). ^{1}H NMR (DMSO-d_6): δ 8.18 (d, 1H, J = 7.3, NH-Ala), 8.04 (m, 1H, NH-CH_3), 7.25 (m, 5H, Ph), 4.19 (q, 1H, J = 7.3, α-H, Ala), 3.78 (m, 1H, H-4), 3.37 (d, 1H, J = 13.8, 2-CH_2,

2-BnAze), 3.19 (m, 1H, H-4), 2.98 (d, 1H, J = 13.8, 2-CH_2, 2-BnAze), 2.67 (d, 3H, J = 4.6, CH_3-NH), 2.23 (m, 1H, H-3), 2.00 (m, 1H, H-3), 1.83 (s, 3H, CH_3-CO), 1.14 (d, 3H, J = 7.3, β-H, Ala). ^{13}C NMR ($CDCl_3$): δ 174.1 (2-CO), 173.9 (CO-Ala), 169.0 (CO-CH_3), 134.6 (C-Ph), 130.3, 128.5, 127.4 (CH-Ph), 75.7 (2-C), 46.1 (4-C), 45.0 (α-C, Ala), 39.6 (2-CH_2, 2-BnAze), 26.3 (CH_3-NH), 23.3 (CH_3-CO), 22.8 (3-C), 18.1 (β-C, Ala). ES-MS: 318.3 $[M + 1]^+$, 340.3 $[M + Na]^+$. Anal. Calcd for $C_{17}H_{23}N_3O_3$: C, 64.33; H, 7.30; N, 13.24. Found: C, 64.52; H, 7.22; N, 13.33.

Piv-Ala-2(*S*)-BnAze-NHMe (12c). Solid (mp 102−104 °C), 65% yield (from **8c**, Method A). Eluent: MeOH/CH_2Cl_2 (1:100). $[\alpha]_D$ = +13.0 (*c* 0.18, $CHCl_3$). HPLC: t_R = 2.61 min (Novapak, A:B = 40:60). ^{1}H NMR ($CDCl_3$): δ 8.16 (m, 1H, NH-CH_3), 7.36−7.12 (m, 5H, Ph), 6.49 (d, 1H, J = 7.2, NH-Ala), 4.31 (q, 1H, J = 7.2, α-H, Ala), 3.64 (m, 1H, H-4), 3.58 (d, 1H, J = 13.8, 2-CH_2, 2-BnAze), 3.16 (m, 1H, H-4), 2.97 (d, 1H, J = 13.8, 2-CH_2, 2-BnAze), 2.87 (d, 3H, J = 4.7, CH_3-NH), 2.75 (ddd, 1H, J = 12.1, 9.8, 7.3, H-3), 2.24 (ddd, 1H, J = 12.1, 9.2, 4.9, H-3), 1.27 (s, 9H, CH_3-*t*Bu), 1.26 (d, 3H, J = 7.2, β-H, Ala). ^{1}H NMR (DMSO-d_6): δ 8.04 (m, 1H, NH-CH_3), 7.49 (d, 1H, J = 7.1, NH-Ala), 7.31−7.18 (m, 5H, Ph), 4.22 (q, 1H, J = 7.1, α-H, Ala), 3.79 (m, 1H, H-4), 3.38 (d, 1H, J = 13.5, 2-CH_2, 2-BnAze), 3.20 (m, 1H, H-4), 3.02 (d, 1H, J = 13.5, 2-CH_2, 2-BnAze), 2.68 (d, 3H, J = 4.8, CH_3-NH), 2.26 (m, 1H, H-3), 2.02 (m, 1H, H-3), 1.18 (d, 3H, J = 7.1, β-H, Ala), 1.11 (s, 9H, CH_3-*t*Bu). ^{13}C NMR ($CDCl_3$): δ 181.0 (CO-*t*Bu), 174.4 (CO-Ala), 174.0 (2-CO), 134.6 (C-Ph), 130.2, 128.5, 127.4 (CH-Ph), 75.7 (2-C), 46.0 (4-C), 45.0 (α-C, Ala), 39.5 (2-CH_2, 2-BnAze), 38.6 (C-*t*Bu), 27.4 (CH_3-*t*Bu), 26.3 (CH_3-NH), 22.7 (3-C), 18.1 (β-C, Ala). ES-MS: 360.3 $[M + 1]^+$, 382.3 $[M + Na]^+$. Anal. Calcd for $C_{20}H_{29}N_3O_3$: C, 66.83; H, 8.13; N, 11.69. Found: C, 66.71; H, 8.22; N, 11.78.

Molecular Modeling Studies. The initial conformations were built using the library of fragments available in the molecular modeling program Insight II (version 2000.1, Biosym Tech., San Diego, CA). The calculations were run using the AMBER force field implemented in Discover, with a distance-dependent dielectric constant (4*r*) and a cutoff of 16 Å. After heating at 1000 K and equilibrated during 100 ps, the structure was cooled slowly to 300 K in steps. In each step the temperature was lowered by 100 K, and the system was kept at this temperature during 100 ps. After cooling at 300 K, the resulting conformations were energy-refined using a conjugated gradient algorithm until the root-mean-square (rms) value of the potential energy gradient was less than 0.001 kcal Å^{-1}mol^{-1}. The conformer was stored and used to start a new simulation at high temperature. This procedure produced samples of 1000 energy-minimized conformations, which were compared to each other to eliminate the identical ones. The protocol was run three times, employing different starting conformations, but the families of conformers obtained were independent of the initial conformation.

X-ray Diffraction Studies. Preparation of Single Crystals for X-ray Diffraction Analysis. Pure peptide derivative **12c** (9 mg) was dissolved in hot hexane (7 mL) and left to cool, resulting in spontaneous crystallization after 11 days at 4 °C.

X-ray Diffraction Crystalograhic Analysis. X-ray diffraction data were obtained in a Bruker-Nonius KappaCCD2000 single-crystal diffractometer equipped with a normal focus sealed tube X-ray source with graded mirror monochromated Cu Kα radiation (λ = 1.5418 Å). Data collection and data reduction were carried out with HKL Denzo and Scalepack.[52] The structure was solved by direct methods Sir2004[53] and was refined by full-matrix least-squares procedures, with anisotropic thermal parameters in the last cycles of refinement for all non-H atoms. H atoms were introduced into the geometrically calculated positions, and refined riding on the corresponding parent atoms. Weighted R factors (w*R*) and all goodness-of-fit (*S*) are based on *F*2, and conventional *R* factors are based on *F*. For refinement, SHELXTL software package (Shelxtl Bruker Axs Inc., 5465 East Cheryl Parkway, Madison, WI 53711-5373, USA) was used.

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Supporting Information Available: Conformational studies, *cis*/*trans* isomery of compounds **7**−**12**. Experimental procedures and analytical and spectroscopic data of compounds **1b**, **2a,b**, **3a,b**, **4a**−**c**, **5b**, **6c**, **7a,b**, **8a**, **9a,b**, **10a,b**, **11b** and **12a,b**. Relevant conformational parameters of dipeptide derivatives **9a,b** and **11a,b**. NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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