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Tetrahedron 61 (2005) 1871–1883

Synthesis and conformational analysis of 18-membered Aib-containing cyclohexapeptides

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Received 11 October 2004; revised 29 November 2004; accepted 2 December 2004

Available online 13 January 2005

Abstract—The synthesis and conformational analysis of two Aib-containing cyclic hexapeptides, *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) **1** and *cyclo*(Leu-Aib-Phe-Gly-Aib-Aib) **2**, is described. The linear precursors of **1** and **2** were prepared using solution phase techniques, and the cyclization efficiency of three different coupling reagents (HATU, PyAOP, DEPC) was examined. The success of the cyclization was found to be reagent dependent. Solid-state conformational analysis of **1** and **2** was performed by X-ray crystallography and has revealed some unusual features as all three Aib residues of **1** assume nonhelical conformations. Furthermore, the residue Aib^4 adopts an extended conformation ($\phi = -175.9(3)°$, $\psi = +178.6(2)°$), which is, to the best of our knowledge, the first observation of an Aib residue adopting an extended conformation in a cyclopeptide. The structure of **1** is also a rare example in which an Aib residue occupies the $(i+1)$ position of a type II′ β-turn, stabilized by a bifurcated hydrogen bond. The cyclic peptide **2** adopts a more regular conformation in the solid state, consisting of two fused β-turns of type I/I′, stabilized by a pair of intramolecular hydrogen bonds. In addition, the conformational study of the cyclic peptide **1** in DMSO-d_6 by NMR spectroscopy and molecular dynamics simulations revealed a structure, which is very similar to its structure in the crystalline state.
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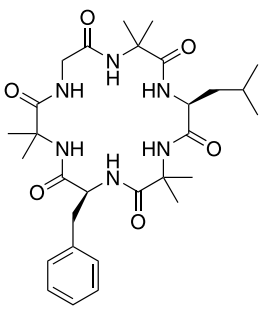
1. Introduction

Cyclic peptides continue to be challenging targets for chemical synthesis.[1] As the synthesis of linear peptides generally proceeds well, the key step for the chemical synthesis of cyclic peptides is usually the cyclization reaction. In particular, the cyclization of small peptides of less than seven amino acid residues is often difficult.[2] Incorporation of turn-inducing elements such as Gly, Pro, D-amino acids and *N*-alkylated amino acids into the peptide backbone is known to improve cyclization yields.[3] Although conformational constraints are usually introduced into peptides through cyclization, cyclic peptides can still possess a remarkable flexibility.[4,5] Thus, the incorporation of sterically hindered C(2)-tetrasubstituted α-amino acids into the peptide backbone leads to more rigid compounds. In addition, cyclic penta- and hexapeptides are often chosen for the synthesis of model cyclopeptides, since larger cyclic peptides already exhibit greater flexibility.[6] Conformation-constrained cyclic peptides may have enhanced metabolic stability, receptor selectivity, and bioavailability, all of which may lead to useful medicinal properties.

Our previous successful synthesis of cyclic hexapeptides containing several Aib (α-aminoisobutyric acid) residues and two Gly residues in positions 1 and 4 of the peptide backbone[7,8] prompted us to investigate the cyclization of hexapeptides containing only one Gly residue as the turn-inducing element. Here, we describe the synthesis of two cyclic hexapeptides *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (**1**) and *cyclo*(Leu-Aib-Phe-Gly-Aib-Aib) (**2**), composed of three protein amino acids, i.e. Gly, Leu, Phe and three α-aminoisobutyric acids. The crystal structures of both cyclic peptides were examined by X-ray diffraction in order to study the influence of the Aib residues on the conformation of the backbone of the cyclic hexapeptides. A NMR-based structure determination of **1** in solution was also performed in the present study.



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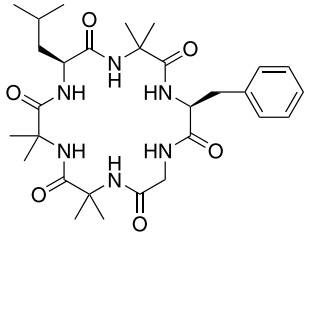

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Keywords: Cyclic peptides; Peptide synthesis; α-Aminoisobutyric acid; Peptide conformation.
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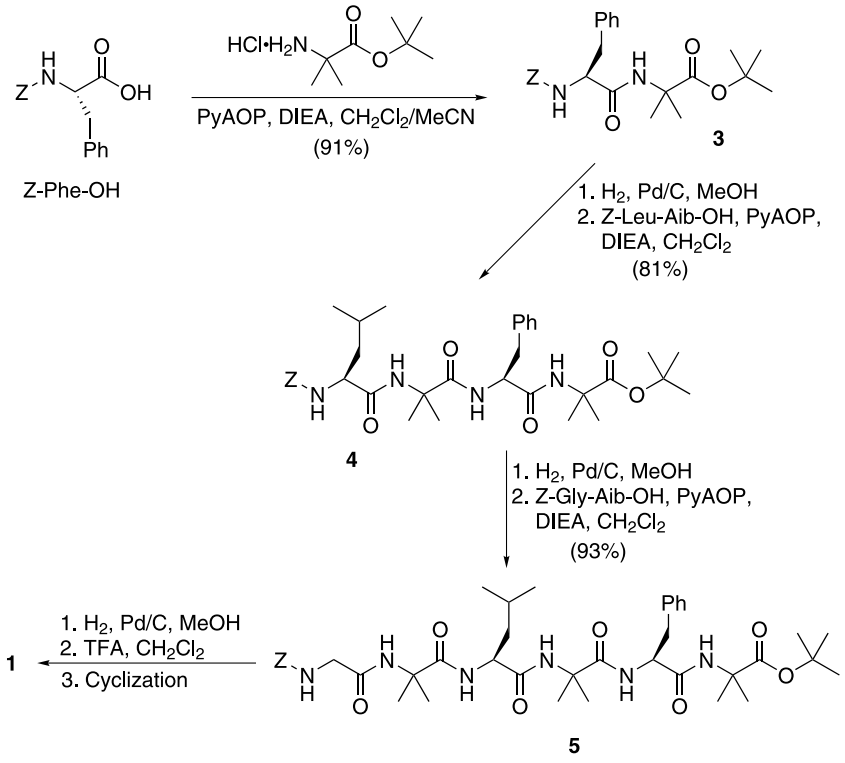
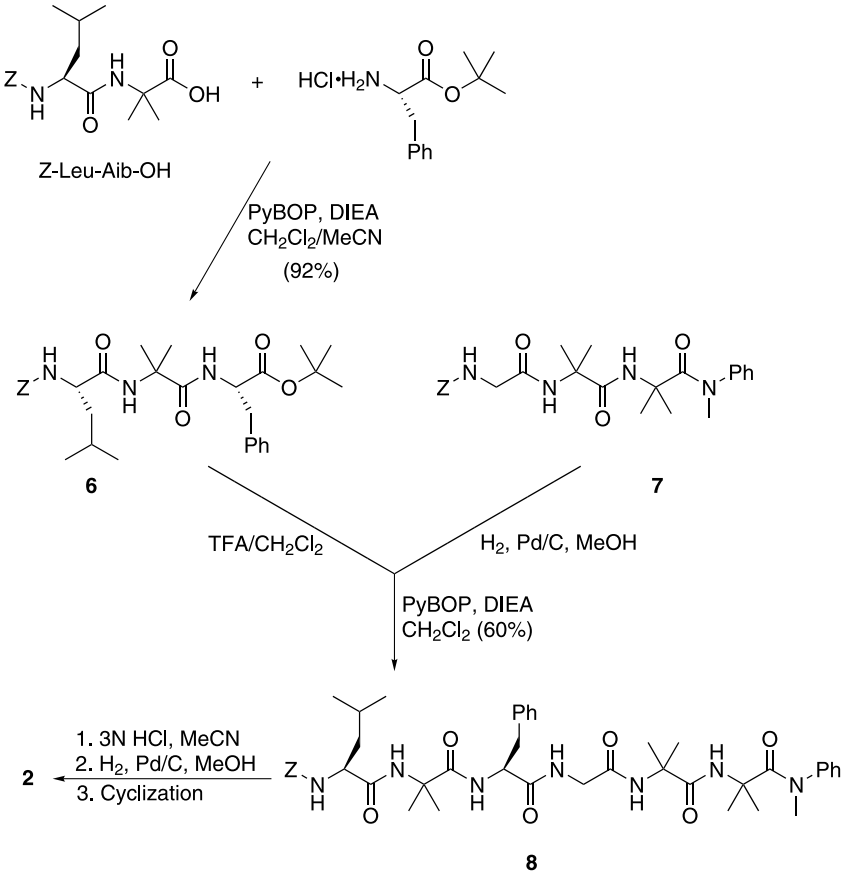
doi:10.1016/j.tet.2004.12.012



Scheme 1.



Scheme 2.

Table 1. Conditions used for the cyclization of Aib-containing hexapeptides **5** and **8**

Cyclic peptide	Cyclization method	Reaction time	Yield (%)
Cyclo(Gly-Aib-Leu-Aib-Phe-Aib) **1**	HATU/HOAt (3 equiv)	1 day	24
	PyAOP/HOAt (5 equiv)	3 days	31
	DEPC (10 equiv)	6 days	53
Cyclo(Leu-Aib-Phe-Gly-Aib-Aib) **2**	HATU/HOAt (3 equiv)	3 days	16
	DEPC (10 equiv)	6 days	33
	PyAOP/HOAt (3 equiv)	3 days	48

2. Results and discussion

2.1. Preparation and cyclization of linear hexapeptides

The linear hexapeptides Z-Gly-Aib-Leu-Aib-Phe-Aib-O*t*Bu (**5**) and Z-Leu-Aib-Phe-Gly-Aib-Aib-N(Me)Ph (**8**) were synthesized by solution-phase methods as shown in Schemes 1 and 2. A [2+2+2]-fragment condensation was chosen in the case of **5**. At first, the dipeptide Z-Phe-Aib-O*t*Bu (**3**) was prepared by coupling Z-Phe-OH with HCl·H-Aib-O*t*Bu using PyAOP as the coupling reagent. Then, **3** was *N*-deprotected to give H-Phe-Aib-O*t*Bu by means of catalytic hydrogenation. The PyAOP-mediated coupling of the latter with Z-Leu-Aib-OH[9] afforded tetrapeptide Z-Leu-Aib-Phe-Aib-O*t*Bu (**4**) in high yield. Removal of the Z protecting group of **4**, and coupling of the resulting H-Leu-Aib-Phe-Aib-O*t*Bu with Z-Gly-Aib-OH in the presence of PyAOP afforded the linear hexapeptide **5**.

For the synthesis of the linear hexapeptide **8**, a convergent [3+3] strategy was employed. Thus, the tripeptide Z-Leu-Aib-Phe-O*t*Bu (**6**) was prepared by coupling Z-Leu-Aib-OH with the hydrochloride of H-Phe-O*t*Bu using PyBOP as the coupling reagent. Treatment of **6** with TFA in CH_2Cl_2, followed by the reaction with H-Gly-Aib-Aib-N(Me)Ph, which was obtained by deprotection of Z-Gly-Aib-Aib-N(Me)Ph (**7**),[7] led, in the presence of PyAOP, to the hexapeptide **8** in moderate yield.

The linear hexapeptides Z-Gly-Aib-Leu-Aib-Phe-Aib-O*t*Bu (**5**) and Z-Leu-Aib-Phe-Gly-Aib-Aib-N(Me)Ph (**8**) were then deprotected at the N- and C-terminus and treated with the coupling reagents HATU, PyAOP and DEPC to investigate the cyclization tendency of each peptide. All of the cyclization reactions were performed in diluted DMF solutions (10^{-4}–10^{-3} M) using a large excess of coupling reagent and base (DIEA). The yields of the cyclization reactions are shown in Table 1. In the first attempts we used HATU as the activating agent, since it has proven to be versatile and highly efficient.[2] However, the cyclohexapeptides **1** and **2** were obtained only in relatively low yields. One explanation for the less efficient macrolactamization than expected could be that HATU participated in a side reaction at the amino terminus to give a guanidino derivative. This side reaction is known to occur when an excess of the aminium salt based coupling reagents is used.[10] To avoid this problem, phosphonium reagents such as PyBOP and PyAOP are recommended.[11] Thus, PyAOP together with HOAt was employed in the cyclization step, leading to **1** in moderate yield while **2** was obtained in good yield. Next, the macrolactamization ability of the organophosphorus reagent DEPC was tested. Because of the slower reaction rate under the DEPC/DIEA conditions, reaction times of up to six days were used. This time, the cyclic hexapeptide **1** was obtained in good yield, while cyclopeptide **2** was isolated in moderate yield. As is evident from these results, the success of the cyclization is dependent upon the choice of the cyclization reagent. However, **1** and **2** were obtained in remarkably similar overall moderate (30%) to good yields (50%). Comparing the best cyclization protocols for **1** and **2** it was surprising that the lactamization between the less hindered pair H_2N-Gly and Aib-CO proceeded only slightly better than that between NH_2 of the sterically demanding Leu residue and Aib-CO. It appears that the conformation or (and) sequence of the linear precursor played a more important role than the size of the residue at the N-terminus.

The cyclic structures of **1** and **2** were established by standard two-dimensional NMR techniques. The assignment of all H- and C-signals was possible by using 2D HSQC and HMBC spectra. A combination of these two types of spectra allowed the complete assignment of the amide NH, CO and C(α) signals of all residues, as well as enabling the signals of different Aib residues to be distinguished. In addition, selected ROESY correlations observed in DMSO-d_6 solution are shown in Figure 1.

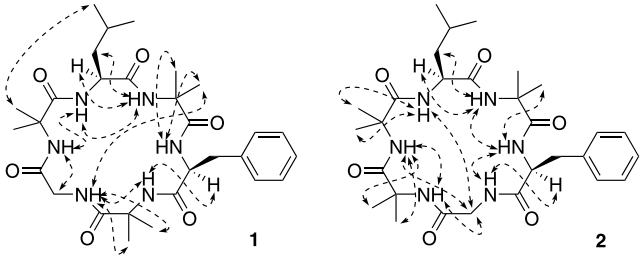


Figure 1. Selected ROESY correlations for compounds **1** and **2** in DMSO-d_6.

2.2. Solid state conformation

Cyclic peptides are frequently found among natural products and exhibit a wide range of biological activities.[12] Therefore, their conformations have been studied extensively both in the solid state and in solution, since the chemical properties and biological activities of such structures are known to be closely related to their molecular conformation.[13–15] The crucial determinants of the conformation of cyclic peptides are the turns (β, γ) and intramolecular hydrogen bonds. Cyclic hexapeptides have been used as model peptides for β-turns since these peptides, due to geometric factors, generally adopt a conformation with two β-turns, stabilized by a pair of two intramolecular hydrogen bonds between residues i and $i+3$.[16] Turns do not necessarily contain hydrogen bonds,

but the lack of hydrogen bonds generally results in distorted or unstable structures.

By extensive crystallographic studies, α-amino isobutyric acid (Aib) has been shown to favor left- or right-handed 3_{10}/α-helical conformations in a wide variety of acyclic peptides of differing lengths and sequences.[17–20] Indeed, Aib residues with very few exceptions almost invariably adopt conformations with ϕ and ψ values near $\pm(60\pm20)^\circ$ and $\pm(30\pm20)^\circ$, respectively. In addition, theoretical calculations show the presence of minima in a semiextended region of the ϕ, ψ space ($\phi=\pm(60\pm20)^\circ$, $\psi=\pm(120\pm20)^\circ$).[17]

There are relatively few reports concerning crystallographic studies of Aib residues incorporated into cyclic peptides,[21–25] so the structural information about the conformational preferences of Aib residues in cyclic molecules is rather scarce. The cyclic tetrapeptide dehydrochlamydocin[21] and the cyclic pentapeptide *cyclo*(Phe-Phe-Aib-Leu-Pro)[22] both have the Aib residue at the center of a γ-turn with unexpected values of the torsion angles ϕ, ψ that lie in the nonhelical conformational space. The torsion angles (ϕ, ψ) of the Aib residues in the crystal structures of *cyclo*(Gly-Aib-Gly)$_2$,[23] [Aib^{5,6}-D-Ala^8]cyclolinopeptide A,[24] and *cyclo*(Pro-Phe-Phe-Aib-Leu)$_2$[25] all lie well inside the 3_{10}/α-helical region of the conformational space, as is observed in linear peptides.

In our research group, we have synthesized several Aib-containing cyclopentapeptides,[19,20] and the crystal-structures of three of them have been established by X-ray crystallography. The structures of *cyclo*[Gly-(*RS*)-Phe(2Me)-Aib-Aib-Gly][19] and *cyclo*[Gly-Aib-(*RS*)-Phe(2Me)-Aib-Gly][20] are very similar and have a β-turn, which is stabilized by a hydrogen bond between NH of Gly^1 and CO of Phe(2Me)^3 and CO of Aib^3, respectively. Whereas in the structure of *cyclo*[Gly-Aib-(*RS*)-Phe(2Me)-Aib-Gly] the torsion angles of both Aib residues show values typical for the helical region (*cyclo*[Gly-Aib-(*R*)-Phe(2Me)-Aib-Gly]: $\phi(\text{Aib}^2)=+50.4(7)^\circ$, $\psi(\text{Aib}^2)=+42.9(6)^\circ$, $\phi(\text{Aib}^4)=-47.2(7)^\circ$, $\psi(\text{Aib}^4)=-46.4(6)^\circ$; *cyclo*[Gly-Aib-(*S*)-Phe(2Me)-Aib-Gly]: $\phi(\text{Aib}^2)=-52.9(7)^\circ$, $\psi(\text{Aib}^2)=-31.2(7)^\circ$, $\phi(\text{Aib}^4)=+50.0(7)^\circ$, $\psi(\text{Aib}^4)=+44.9(7)^\circ$), those of Aib^3 of *cyclo*[Gly-(*RS*)-Phe(2Me)-Aib-Aib-Gly] do not correspond with the helical conformational space ($\phi=-159.7(2)^\circ$, $\psi=+166.3(2)^\circ$). In the asymmetric unit of the crystal structure of the third cyclopentapeptide, i.e. *cyclo*[Gly-(*R*)-Phe(2Me)-Pro-Aib-Phe],[20] there are two independent molecules with quite different conformations. One of the molecules forms a β-turn with a Aib$^4\rightarrow$Gly^1 hydrogen bond, whereas the other molecule is characterized by a γ-turn (NH(Aib^4)$\rightarrow$CO(Pro^3) hydrogen bond) and an α-turn (NH(Phe^5)–CO(Gly^1) hydrogen bond). In the molecule containing a β-turn, Aib^4 shows ϕ, ψ values that belong to the nonhelical conformational space ($\phi=+154.8(4)^\circ$, $\psi=-51.0(4)^\circ$).

As a part of our investigation of the synthesis of cyclic hexapeptides containing Aib residues,[7,26] we have been interested in the conformations of these cyclic molecules in order to estimate if the incorporation of several Aib residues into cyclic hexapeptide structures stabilizes certain types of turns. Previously, we have investigated the cyclization and conformation of hexapeptides containing two or three Aib residues, and two Gly residues in positions 1 and 4 of the peptide backbone.[7] The crystal structures of two cyclic peptides, *cyclo*(Gly-Aib-Aib-Gly-Aib-Phe) and *cyclo*(Gly-(*S*)-Phe(2Me)-Aib-Gly-Aib-Phe), showed that these molecules have two fused β-turns. The observed β-turns were stabilized by intramolecular hydrogen bonds between the NH of Gly^1 and the C=O of Gly^4 and between the NH of Gly^4 and C=O of Gly^1. Different types of β-turn conformations, i.e. I, I′ and III′, have been observed depending on the sequence, with Aib residues occupying positions ($i+1$) and/or ($i+2$) of the turns.

The solid-state conformations of the new cyclohexapeptides *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (**1**) and *cyclo*(Leu-Aib-Phe-Gly-Aib-Aib) (**2**) were examined by X-ray crystallography. Crystals of **1** suitable for the X-ray analysis were obtained from a mixture of MeOH/*i*-PrOH/$CHCl_3$ and acetone, and those of **2** were grown from MeOH/EtOH/*i*-PrOH and water. The ORTEP plots[27] of the molecules with the atom numbering schemes are presented

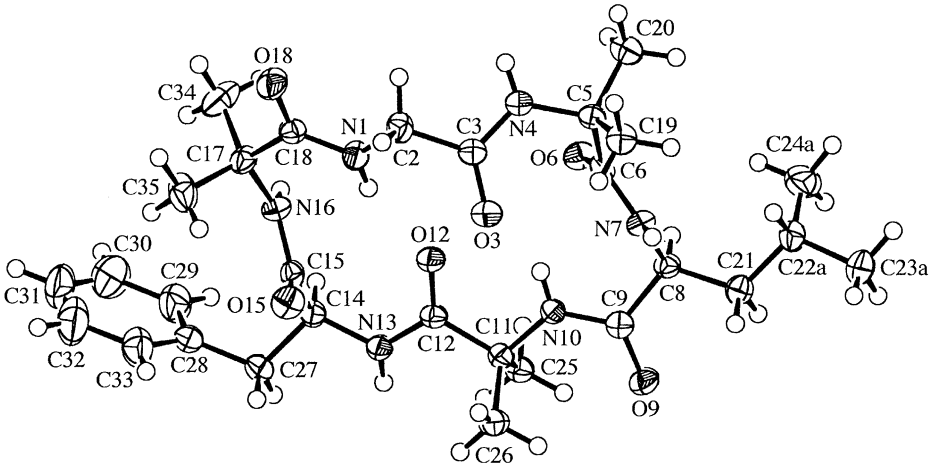


Figure 2. *ORTEP plot*[27] of the molecular structure of **1** (50% Probability ellipsoids, arbitrary numbering of atoms, only one of the disordered arrangements of the Leu side chain is shown).

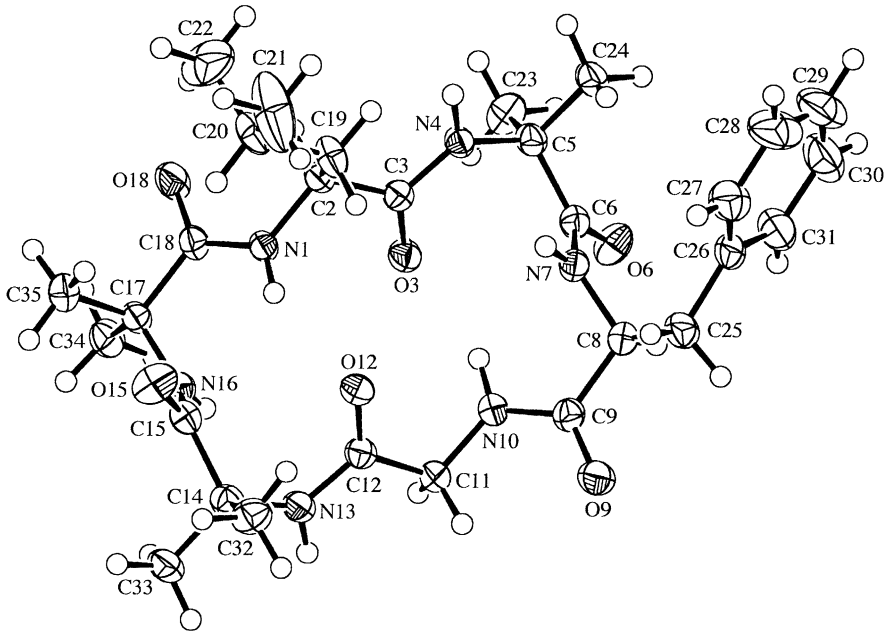


Figure 3. *ORTEP plot*[27] of the molecular structure of **2** (50% Probability ellipsoids, arbitrary numbering of atoms, solvent molecules omitted for clarity).

Table 2. Backbone torsion angles [°] for the crystal structures of **1** and **2**

1		Gly[1]	Aib[2]	Leu[3]	Aib[4]	Phe[5]	Aib[6]
	ϕ	−170.0(3)	+55.2(3)	−90.7(3)	−175.9(3)	−45.4(4)	+76.9(4)
	ψ	+119.6(3)	−130.4(3)	+42.6(3)	+178.6(2)	+127.2(3)	−7.7(4)
	ω	+178.2(2)	−164.7(2)	+166.1(2)	−171.8(2)	+169.1(2)	−178.1(3)
2		Leu[1]	Aib[2]	Phe[3]	Gly[4]	Aib[5]	Aib[6]
	ϕ	−113.0(3)	−57.1(3)	−113.3(3)	+117.8(3)	+56.4(3)	+93.8(3)
	ψ	−171.7(2)	−34.4(3)	+22.8(3)	+169.4(2)	+41.2(3)	−15.3(3)
	ω	−165.6(2)	−171.4(2)	−178.3(2)	+170.3(2)	+172.5(2)	−173.8(2)

in Figures 2 and 3. The isopropyl part of the Leu side chain of **1** is disordered over two almost equally occupied conformations, while the asymmetric unit of **2** contains one molecule of the cyclic peptide plus two water molecules, one disordered EtOH molecule and one disordered *i*-PrOH molecule. Two approximately equally occupied positions were modelled for each of the disordered solvent molecules. The backbone torsion angles of the cyclopeptides are summarized in Table 2 and the hydrogen bonding parameters in Tables 3 and 4.

Surprisingly, all three Aib residues of *cyclo*(Gly[1]-Aib[2]-Leu[3]-Aib[4]-Phe[5]-Aib[6]) (**1**) assume conformations in the nonhelical region of the *Ramachandran* diagram (Table 2). The deviation of the backbone conformation from the helical region is less pronounced at Aib[6]. The residue Aib[2] adopts a rare semiextended conformation with torsion angles ϕ, ψ almost identical to those previously reported for a cyclic hexapeptide having a disulfide linkage.[28] Interestingly, very similar ϕ, ψ values were also observed for one Aib residue in the linear tetrapeptide Boc-Leu-Aib-Phe-Aib-OMe, which was reported to form a continuous hydrogen-bonded supramolecular helix.[29] The residue Aib[4] adopts a fully extended conformation, so far known to be characteristic of the higher homologs of Aib, like α,α-diethylglycine (Deg),[30–32] and α,α-dipropylglycine (Dpg).[33,34] The torsion angles ϕ and ψ of the Phe and Aib[6] residues in the Aib[4]-Phe[5]-Aib[6]-Gly[1] sequence show values close to those for a type II β-turn with an intramolecular 1←4 hydrogen bond (N(1)–H…O(12))

Table 3. Hydrogen bonding parameters for *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (**1**)

Donor D-H	Acceptor A	Distance [Å] D-H	Distance [Å] H…A	Distance [Å] D…A	Angle [°] D-H…A
N(1)–H(1)	O(12)	0.81(3)	2.34(3)	3.126(4)	163(3)
N(4)–H(4)	O(3[i])	0.84(3)	2.30(3)	3.131(4)	172(3)
N(7)–H(7)	O(18[ii])	0.94(3)	2.00(3)	2.877(3)	154(3)
N(10)–H(10)	O(12)	0.83(3)	2.17(3)	2.604(3)	113(3)
N(10)–H(10)	O(6)	0.83(3)	2.38(3)	3.083(3)	143(3)
N(13)–H(13)	O(6[iii])	0.87(3)	2.11(3)	2.972(3)	171(3)
N(16)–H(16)	O(9[iv])	0.94(3)	1.95(4)	2.867(3)	163(3)

Primed atoms refer to the molecule in the following symmetry-related positions: [i] $2-x, -1/2+y, 1/2-z$; [ii] $2-x, 1/2+y, 1/2-z$; [iii] $1-x, 1/2+y, 1/2-z$; [iv] $1-x, -1/2+y, 1/2-z$.

Table 4. Hydrogen bonding parameters for *cyclo*(Leu-Aib-Phe-Gly-Aib-Aib) (**2**)

Donor D-H	Acceptor A	Distance [Å] D-H	Distance [Å] H…A	Distance [Å] D…A	Angle [°] D-H…A
N(1)–H(1)	O(12)	0.87(4)	2.24(4)	3.042(3)	154(3)
N(4)–H(4)	O(44^{i})	0.89(4)	1.90(4)	2.782(3)	171(3)
N(7)–H(7)	O(38a)	0.89(4)	2.21(4)	2.92(1)	136(3)
N(7)–H(7)	O(38b)	0.89(4)	2.18(4)	2.87(1)	135(3)
N(10)–H(10)	O(3)	0.93(4)	2.18(4)	3.078(3)	161(3)
N(13)–H(13)	O(43)	0.88(4)	1.91(4)	2.787(3)	176(3)
N(16)–H(16)	O(42a)	0.73(3)	2.48(3)	3.10(1)	145(3)
N(16)–H(16)	O(42b)	0.73(3)	2.38(3)	2.99(1)	141(3)
O(38a)–H(381)	O(12)	0.84	1.91	2.75(2)	173
O(38b)–H(382)	O(12)	0.84	2.26	2.87(2)	129
O(42a)–H(421)	O(3)	0.84	2.15	2.79(2)	132
O(42b)–H(422)	O(3)	0.84	1.96	2.74(2)	154
O(43)–H(431)	O(15^{ii})	0.98(6)	1.83(6)	2.805(3)	174(4)
O(43)–H(432)	O(18^{iii})	0.79(5)	1.96(5)	2.750(3)	177(4)
O(44)–H(441)	O(6^{iv})	0.87(6)	1.89(6)	2.754(3)	171(5)
O(44)–H(442)	O(9)	0.95(5)	1.80(5)	2.734(3)	168(4)

Primed atoms refer to the molecule in the following symmetry-related positions: $^{i}-1+x, y, z$; $^{ii}1-x, -1/2+y, 2-z$; $^{iii}1+x, y, z$; $^{iv}1-x, 1/2+y, 1-z$.

involving the NH of Gly^1 and the C=O of Aib^4 (Tables 2 and 3). In contrast, the backbone conformation of the sequence Gly^1-Aib^2-Leu^3-Aib^4 cannot be strictly categorized. It could best be described as a distorted type II′ β-turn, with values for ϕ_{i+1} (+55.2(3)°), ψ_{i+1} (−130.4(3)°) and ϕ_{i+2} (−90.7(3)°) being close to the ideal values ($\phi_{i+1}=+(60\pm30)°$; $\psi_{i+1}=-(120\pm30)°$, $\phi_{i+2}=-(80\pm30)°$) for this type of turn, and ψ_{i+2} (+42.6(3)°) deviating largely from the ideal value ($\psi_{i+2}=0\pm50°$) (Table 2). The residue Aib^2 is obviously forced to assume the conformation of a D-amino acid as it prefers the $(i+1)$ position of a β-turn of type II′.[16] As a consequence of the large deviation of ψ_{i+2} from ideality, no 1←4 intramolecular hydrogen bond is observed between the NH of Aib^4 and C=O of Gly^1. However, the extended conformation of Aib^4 gives rise to an intramolecular hydrogen bond between the NH and C=O groups within this residue (N(10)–H…O(12), Table 3), which is unusual and has only infrequently been inferred from crystal structure data of some dipeptides.[17] Furthermore, the NH group of Aib^4 acts not only as a donor for C=O of Aib^4, but also as a donor for the carbonyl group of Aib^2, and is thus involved in inverse bifurcation.[35]

The cyclic peptide *cyclo*(Leu^1-Aib^2-Phe^3-Gly^4-Aib^5-Aib^6) (**2**) was found to possess a more regular structure with two fused β-turns stabilized by two intramolecular hydrogen bonds, one between C=O of Leu^1 and NH of Gly^4 (N(10)–H…O(3), Table 4) and the other between C=O of Gly^4 and NH of Leu^1 (N(1)–H…O(12)). Furthermore, the conformation of only one of the Aib residues, Aib^6, shows slight deviation from the helical region of the conformational space. The values of torsion angles ϕ and ψ reveal the presence of a type I β-turn across Leu^1-Aib^2-Phe^3-Gly^4 and a type I′ β-turn spanning the residues Gly^4-Aib^5-Aib^6-Leu^1 (Table 2).

2.3. Solution conformational analysis

The conformation of the cyclic hexapeptide *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (**1**) in DMSO-d_6 solution has been determined by ^{1}H NMR spectroscopy. The structure calculation was performed by restrained molecular dynamics in torsion angle space by applying the simulated annealing protocol implemented in the program DYANA.[36] The NOE intensities were calibrated with the tools of the program, and yielded an input of 45 upper-distance limits (11 intra-residual, 29 sequential and 5 medium/long-range) (Tables 5–7). The final calculation was started with 100 randomized conformers, and a bundle of 20 DYANA conformers with the lowest target function was selected for structure analysis and visualization with the program MOLMOL.[37] The results of DYANA calculations for **1** are shown in Figure 4, and the observed average backbone torsion angles (ϕ, ψ) are listed in Table 8.

As is evident from Figure 4 and Table 8, **1** is well structured in solution and with a mean RMSD value of the backbone atoms of 0.3 Å very similar to the backbone conformation found in the crystal structure, although some torsion angles

Table 5. Intraresidual upper distance restraints derived from integration of ROESY cross-peak volumes for the cyclic peptide *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (**1**)

Residue	Atom	Residue	Atom	Distance [Å]
Gly^1	HN	Gly^1	HA1	2.87
Leu^3	HN	Leu^3	HB2	2.77
Leu^3	HA	Leu^3	HB2	2.68
Leu^3	HA	Leu^3	HB3	2.62
Leu^3	HA	Leu^3	QD1	3.64
Leu^3	HA	Leu^3	QD2	5.69
Phe^5	HN	Phe^5	HA	2.83
Phe^5	HN	Phe^5	HB2	2.99
Phe^5	HN	Phe^5	HB3	3.27
Phe^5	HA	Phe^5	HB2	2.65
Phe^5	HA	Phe^5	HB3	2.71

Table 6. Sequential upper distance restraints derived from integration of ROESY cross-peak volumes for the cyclic peptide *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (**1**)

Residue	Atom	Residue	Atom	Distance [Å]
Gly^1	HN	Aib^2	HN	4.69
Gly^1	HN	Aib^6	HN	3.24
Gly^1	HN	Aib^6	QB1	4.74
Gly^1	HN	Aib^6	QB2	5.34
Gly^1	HA1	Aib^2	HN	2.83
Gly^1	HA2	Aib^2	HN	2.71
Aib^2	HN	Leu^3	HN	3.42
Aib^2	QB1	Leu^3	HN	4.55
Aib^2	QB1	Leu^3	HA	6.38
Aib^2	QB1	Leu^3	QD1	7.56
Aib^2	QB1	Leu^3	QD2	7.56
Aib^2	QB2	Leu^3	QD1	7.57
Aib^2	QB2	Leu^3	QD2	7.57
Leu^3	HN	Aib^4	HN	3.11
Leu^3	HA	Aib^4	HN	2.62
Leu^3	HA	Aib^4	QB1	5.88
Leu^3	HB2	Aib^4	HN	3.79
Leu^3	HB3	Aib^4	HN	3.83
Aib^4	QB1	Phe^5	HA	6.04
Aib^4	QB1	Phe^5	QD	8.66
Aib^4	QB2	Phe^5	HN	4.10
Aib^4	QB2	Phe^5	QD	8.67
Phe^5	HN	Aib^6	HN	4.07
Phe^5	HA	Aib^6	HN	2.40
Phe^5	HA	Aib^6	QB2	6.54
Phe^5	HB2	Aib^6	HN	4.82
Phe^5	HB3	Aib^6	HN	4.29
Phe^5	QD	Aib^6	QB2	8.29
Phe^5	QE	Aib^6	QB2	8.67

Table 7. Medium and long range upper distance restraints derived from integration of ROESY cross-peak volumes for the cyclic peptide **1**

Residue	Atom	Residue	Atom	Distance [Å]
Gly^1	HN	Aib^4	HN	4.20
Gly^1	HN	Aib^4	HB1	5.73
Gly^1	HN	Phe^5	HN	4.14
Gly^1	HN	Phe^5	HA	3.61
Aib^4	QB1	Aib^6	HN	6.54

deviate significantly. In particular, the residue Aib^4 assumes almost identical, for an Aib residue unexpected, extended conformations in both the solid state and in the solution. The average conformer exhibits two β-turns, one type I-like β-turn centered at Aib^2-Leu^3 and one type II-like β-turn across Aib^4-Phe^5-Aib^6-Gly^1. The large ^3J(HN, HC(α)) coupling constant of 9 Hz at Leu^3 which correlates to a torsion angle ϕ around $-100°$ provide further support for the occurrence of the type I β-turn in solution. An analysis of the hydrogen-bonding patterns using the final NMR coordinates shows a significant population of intramolecular hydrogen bonding between the carbonyl group of Gly^1 and the NH of the Aib^4 residue, which is contrary to the observation found in the crystal structure where a hydrogen bond is formed between the CO group of Aib^4 and the NH group of Gly^1.

3. Conclusion

In conclusion, we have shown that it is possible to cyclize hexapeptides containing three constrained Aib residues, two rather large proteinogenic amino acid residues (Leu, Phe) and only one Gly residue as a turn-inducing element, in good yields. Since *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (**1**) and *cyclo*(Leu-Aib-Phe-Gly-Aib-Aib) (**2**) have been obtained in similar overall cyclization yields, being 24–53% for **1** and 16–48% for **2**, the choice of coupling reagent apparently played a more important role in the cyclization than the sequence of the linear precursor. In addition, the coupling reagents PyAOP and DEPC proved to be superior to HATU. The structures of **1** and **2** were examined in the solid state by X-ray crystallography in order to gain information about the conformational preferences of Aib residues incorporated into cyclic peptides. A detailed comparison of the crystal structures of **1** and **2** with those obtained previously[7] for *cyclo*(Gly-Aib-Aib-Gly-Aib-Phe) and *cyclo*(Gly-(*S*)-Phe(2Me)-Aib-Gly-Aib-Phe), reveals severe conformational restraints imposed on the peptide backbone of cyclic hexapeptide **1** consisting of alternating Aib and proteinogenic amino acid residues. Thus, all three Aib residues of **1** assume torsion angles well outside the helical region of the conformational space, which is highly uncommon. It appears that the conformational constraint is less pronounced in the other three cyclopeptides having two adjoining Aib residues or one Aib residue adjacent to another α,α-disubstituted amino acid residue such as Phe(2Me) (Phe(2Me)=α-methylphenylalanine). Each of

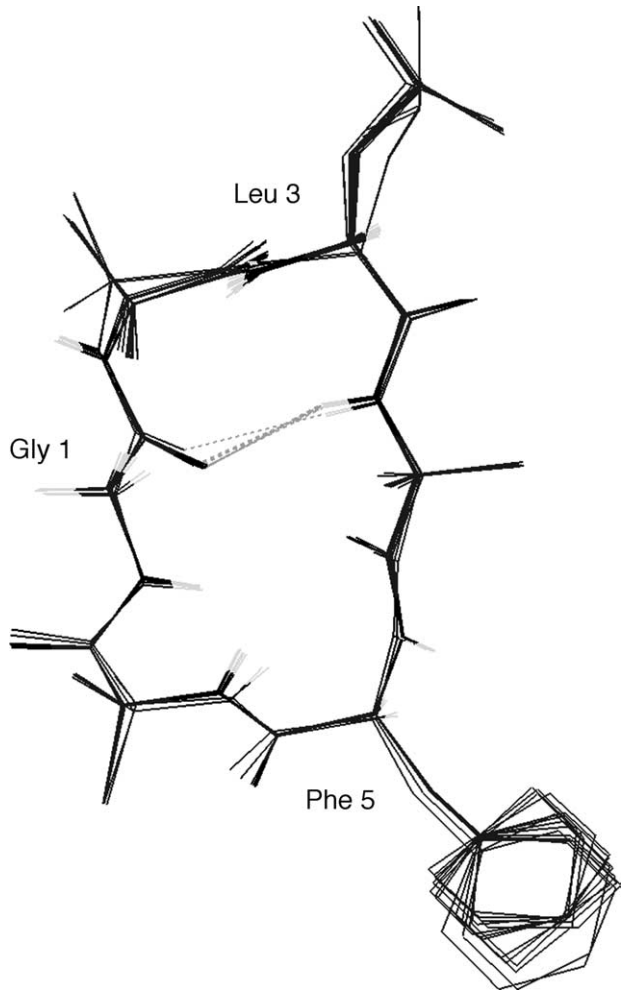


Figure 4. Superimposition of the final 14 NMR structures for **1**.

Table 8. Observed average backbone torsion angles for the cyclic peptide **1** as obtained from the final 14 NMR structures

Residue	ϕ	ψ
Gly[1]	−105.2	+170.0
Aib[2]	−63.8	−26.5
Leu[3]	−123.6	+31.2
Aib[4]	−162.9	−165.3
Phe[5]	−84.6	+88.1
Aib[6]	+80.9	+21.1

these cyclic hexapeptides possesses only one Aib residue that shows slight deviation of torsion angles from the helical region of the conformational space.

4. Experimental

4.1. General

Solvents were purified by standard procedures. Thin-layer chromatography (TLC): Merck TLC aluminium sheets, silica gel 60 F_{254}. Column chromatography (CC): Uetikon-Chemie 'Chromatographiegel' C-560. Mp: Büchi 510 apparatus; uncorrected. IR Spectra: Perkin–Elmer-1600 FT-IR spectrophotometer; in KBr; absorptions in cm^{-1}. ^1H (300 MHz) and ^{13}C NMR (75.5 MHz) spectra: Bruker ARX-300 instrument; ^1H (600 MHz) and ^{13}C NMR (150.9 MHz) spectra of cyclic peptides: Bruker DRX-600 instrument; in (D_6)DMSO at 300 K unless otherwise stated; δ in ppm, coupling constants J in Hz. ROESY spectra were measured with a mixing time of 300 ms. Acquisition parameters of the ROESY experiment of **1**: F_1: ND0 1, TD 512, SFO1 600.1325 MHz, FIDRES 11.252340 Hz, SW 9.600 ppm, FnMODE undefined; F_2: TD 2048, NS 16, SWH 5787.037 Hz, AQ 0.1770836 s, RG 32, d0 0.000003 s, D1 3.000000 s, d11 0.030000 s, d12 0.000020 s. Acquisition parameters of the ROESY experiment of **2**: F_1: ND0 1, TD 512, SFO1 600.1325 MHz, FIDRES 10.783298 Hz, SW 9.200 ppm, FnMODE undefined; F_2: TD 2048, NS 32, SWH 5530.974 Hz, AQ 0.1852796 s, RG 128, d0 0.000003 s, D1 3.000000 s, d11 0.030000 s, d12 0.000020 s. MS: Finnigan SSQ-700 or MAT-90 instrument for CI; Finnigan TSQ-700 triple quadrupole spectrometer for ESI; *m/z* (rel.%). Abbreviations: DEPC: diethylphosphorocyanidate, DIEA: *N*-ethyl-*N*,*N*-diisopropylamine, HATU: *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt: 1-hydroxy-7-azabenzotriazole, PyAOP: (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate, PyBOP: (1*H*-benzotriazol-1-yloxy)-tris(pyrrolidino) phosphonium hexafluorophosphate.

General Procedure A (GP A). To a solution of a Z-protected peptide in MeOH was added Pd/C (10% on activated charcoal) and the mixture was hydrogenated overnight under atmospheric pressure using an H_2-filled balloon. The catalyst was removed by filtration through a pad of celite and the solvent evaporated under reduced pressure. The crude product was further purified by filtration through a short column of SiO_2, dried under vacuum and used directly in the next reaction step.

General Procedure B (GP B). To a solution of an *N*-protected peptide acid (or *N*-protected amino acid) in abs. CH_2Cl_2 (or CH_2Cl_2/MeCN mixture) were added the amino component (1.0 or 1.1 equiv), PyAOP (or PyBOP, 1.1 equiv), and DIEA (2 equiv without and 3 equiv with hydrochloride salts present). The mixture was stirred at rt under N_2 until the starting material was consumed (TLC). The solvent was then evaporated, the residue was dissolved in EtOAc and washed with 5% aq $KHSO_4$ solution, 5% aq $NaHCO_3$ solution and brine. The organic layer was dried ($MgSO_4$), concentrated, purified by CC and dried under high vacuum.

General Procedure C (HATU-mediated Cyclization) (GP C). The free linear hexapeptide was dissolved in abs. DMF (0.7 or 1.5 mM) and cooled to 0 °C in an ice bath. To the solution was added HATU (3 equiv), HOAt (3 equiv, 0.5 M solution in DMF) and DIEA (1% v/v) under stirring. The solution was kept at 0 °C for 2 h and at rt for 3 days. The solvent was removed under reduced pressure, the residue dissolved in EtOAc and washed with 1 M HCl solution, water, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The crude cyclopeptide was further purified by CC.

General Procedure D (DEPC-mediated Cyclization) (GP D). The free linear hexapeptide was dissolved in abs. DMF

(1.5 mM) and the solution was cooled to 0 °C in an ice bath. Then, a solution of 5 equiv of DEPC in abs. DMF (1 ml) was added under stirring, and DIEA (1% v/v) was added slowly over a period of 15 min. The solution was warmed to rt and stirred. The addition of DEPC (2.5 equiv) was repeated after 2 and 4 days, and the reaction mixture was stirred for an additional 2 days. The solvent was then evaporated under reduced pressure, the residue was taken up in EtOAc and washed with 5% aq $KHSO_4$ solution, 5% aq $NaHCO_3$ solution, and brine. The organic phase was then dried ($MgSO_4$) and concentrated to give the crude cyclohexapeptide, which was purified by CC.

General Procedure E (PyAOP-mediated Cyclization) (GP E). The free linear hexapeptide was dissolved in abs. DMF (0.6–1.0 mM) under stirring. Then, PyAOP (3 or 5 equiv), HOAt (3 or 5 equiv, 0.5 M solution in DMF) and DIEA were added at rt and the solution was stirred at rt for an additional 3 days. The solvent was then removed under reduced pressure, the residue dissolved in EtOAc and washed with 10% citric acid solution, 5% $NaHCO_3$ solution, and water. The organic layer was dried (Na_2SO_4), filtered, and concentrated to afford a yellow oil which was purified by CC.

4.2. Preparation of *cyclo*(Gly-Aib-Leu-Aib-Phe-Aib) (1)

4.2.1. *tert*-Butyl *N*-[(benzyloxy)carbonyl]-(*S*)-phenylalanyl-dimethylglycinate (Z-Phe-Aib-O*t*Bu) (3). Z-Phe-OH (0.6 g, 2.0 mmol) was coupled with HCl·H-Aib-O*t*Bu (0.431 g, 2.2 mmol), using PyAOP (1.147 g, 2.2 mmol) and DIEA (0.775 g, 6.0 mmol) in CH_2Cl_2/MeCN (6/4 ml) according to GP B. Reaction time: 20 h at rt. Purification of the crude product by CC (SiO_2, EtOAc/hexane 15:10) afforded 0.798 g (91%) of dipeptide **3**. White powder. Mp 119.5–121.0 °C. IR: 3325s, 3236m, 3065m, 2979m, 2948m, 1727s, 1708s, 1660s, 1544s, 1498m, 1470m, 1455m, 1384m, 1370m, 1291s, 1260s, 1235s, 1215m, 1147s, 1085w, 1065m, 1044m, 1028m, 912w, 853w, 757m, 740m, 700s. ^{1}H NMR: 8.23 (s, NH of Aib); 7.42 (d, J= 8.95 Hz, NH of Phe); 7.34–7.19 (m, 10 arom. H); 4.94 (br s, PhCH_2O); 4.35–4.20 (m, CH(2) of Phe); 2.93–2.92, 2.77–2.73 (2m, CH_2(3) of Phe); 1.35 (s, Me_3C); 1.33, 1.30 (2s, 2Me of Aib). ^{13}C NMR: 172.7, 170.6 (2s, 2 CO); 155.6 (s, CO (urethane)); 138.0, 136.9 (2s, 2 arom. C); 129.1, 128.1, 127.8, 127.5, 127.2, 126.1 (6d, 10 arom. CH); 79.3 (s, Me_3C); 64.9 (t, PhCH_2O); 55.6 (d, C(2) of Phe); 55.3 (s, C(2) of Aib); 37.6 (t, C(3) of Phe); 27.3 (q, Me_3C); 24.6 (q, 2Me of Aib). ESI-MS (NaI+MeOH): 463 (100, [M+Na]$^+$). Anal. calcd for $C_{25}H_{32}N_2O_5$ (440.54): C 68.16, H 7.32, N 6.36; found: C 68.06, H 7.20, N 6.25.

4.2.2. *tert*-Butyl *N*-[(benzyloxy)carbonyl]-(*S*)-leucyl-dimethylglycyl-(*S*)-phenylalanyl-dimethylglycinate (Z-Leu-Aib-Phe-Aib-O*t*Bu) (4). Z-Phe-Aib-O*t*Bu (**3**) (0.475 g, 1.08 mmol) was *N*-deprotected by following GP A (H_2, 55 mg Pd/C, 15 ml MeOH, overnight). The crude product was filtered through a short SiO_2-column with EtOAc/MeOH (15:1) and dried under vacuum to give 0.328 g (99%) H-Phe-Aib-O*t*Bu as a white foam, which was used directly in the next step.

Z-Leu-Aib-OH[9] (0.375 g, 1.07 mmol) was coupled with H-Phe-Aib-O*t*Bu (0.328 g, 1.07 mmol), using PyAOP (0.56 g, 1.1 mmol) and DIEA (0.276 g, 2.14 mmol) in abs. CH_2Cl_2 (10 ml) according to GP B. Reaction time: 20 h at rt. Purification by CC (SiO_2, EtOAc/hexane 20:1) yielded 0.556 g (81%) of tetrapeptide **4** as a white foam. IR: 3325s, 3064w, 3032m, 2979m, 2960m, 2936m, 2872w, 1735s, 1665s, 1535s, 1469m, 1455m, 1385m, 1367m, 1258s, 1222s, 1148s, 1081w, 1029m, 940w, 850w, 788w, 752m, 698m. ^{1}H NMR (CD_3OD): 7.34–7.16 (m, 10 arom. H); 5.12 (s, PhCH_2O); 4.48–4.46, 4.05–3.95 (2m, CH(2) of Leu and CH(2) of Phe); 3.32–3.29 (m, 1H of CH_2(3) of Phe); 2.94 (dd, J=14.2, 10.6 Hz, 1H of CH_2(3) of Phe); 1.80–1.51 (m, CH_2(3) and CH(4) of Leu); 1.44, 1.43, 1.32, 1.16 (4s, 4Me of 2Aib and Me_3C); 0.96, 0.92 (2d, J=6.6 Hz, 2Me(5) of Leu). ^{13}C NMR (CD_3OD): 176.4, 175.3, 174.9, 172.5 (4s, 4 CO); 158.9 (s, CO (urethane)); 139.1, 137.9 (2s, 2 arom. C); 130.1, 129.4, 129.3, 129.0, 128.6, 127.5 (6d, 10 arom. CH); 81.9 (s, Me_3C); 67.7 (t, PhCH_2O); 57.7, 57.6 (2s, 2 C(2) of 2Aib); 55.9 (d, C(2) of Phe); 41.3, 37.8 (2t, C(3) of Phe and C(3) of Leu); 28.1 (q, Me_3C); 25.7 (d, C(4) of Leu); 25.4, 24.9, 24.7, 23.1, 22.1 (5q, 4Me of 2Aib and 2Me(5) of Leu); C(2) of Leu not detectable. ESI-MS (NaI+MeOH): 661 (100, [M+Na]$^+$). Anal. calcd for $C_{35}H_{50}N_4O_7$ (638.80): C 65.81, H 7.89, N 8.77; found: C 65.66, H 8.04, N 8.70.

4.2.3. *tert*-Butyl *N*-[(benzyloxy)carbonyl]-glycyl-dimethylglycyl-(*S*)-leucyl-dimethylglycyl-(*S*)-phenylalanyl-dimethylglycinate (Z-Gly-Aib-Leu-Aib-Phe-Aib-O*t*Bu) (5). Z-Leu-Aib-Phe-Aib-O*t*Bu (**4**) (0.527 g, 0.82 mmol) was *N*-deprotected according to GP A (H_2, 55 mg Pd/C, 10 ml MeOH, overnight). The crude product was filtered through a short SiO_2-column with EtOAc/MeOH (17:1) and dried under vacuum to afford 0.4 g (96%) of H-Leu-Aib-Phe-Aib-O*t*Bu as a white foam. This material (0.4 g, 0.79 mmol) was coupled with Z-Gly-Aib-OH[7] (0.234 g, 0.79 mmol) by following GP B, using PyAOP (0.521 g, 1.0 mmol) and DIEA (0.255 g, 1.6 mmol) in abs. CH_2Cl_2 (10 ml). Reaction time: 20 h at rt. CC (SiO_2, EtOAc/hexane/MeOH 10:7:1) gave 0.576 g (93%) of hexapeptide **5** as a white foam. IR: 3322s, 3065w, 3033w, 2982m, 2959m, 2873w, 1664s, 1534s, 1456m, 1387m, 1368m, 1261m, 1151s, 1082w, 1051w, 979w, 852s, 740w, 699m. ^{1}H NMR (CD_3OD): 7.35–7.15 (m, 6 arom. H); 5.17–5.05 (m, PhCH_2O); 4.42–4.39, 4.15–3.70 (2m, CH(2) of Phe, CH(2) of Leu and CH_2(2) of Gly); 3.35–2.80 (m, CH_2(3) of Phe); 1.90–1.47 (m, CH_2(3) and CH(4) of Leu); 1.45, 1.44, 1.38, 1.18 (4s, 6Me of 3Aib and Me_3C); 0.94, 0.89 (2d, J=6.3 Hz, 2Me(5) of Leu). ^{13}C NMR (CD_3OD): 177.1, 177.0, 174.9, 174.5, 172.8, 172.1 (6s, 6 CO); 159.5 (s, CO (urethane)); 139.3, 137.9 (2s, 2 arom. C); 130.1, 129.5, 129.3, 129.1, 128.6, 127.5 (6d, 10 arom. CH); 81.8 (s, Me_3C); 67.8 (t, PhCH_2O); 57.9, 57.8, 57.7 (3s, 3 C(2) of 3Aib); 56.5, 54.1 (2d, C(2) of Phe and C(2) of Leu); 45.4, 39.9, 37.5 (3t, C(2) of Gly, C(3) of Phe and C(3) of Leu); 28.1 (q, Me_3C); 26.1 (d, C(4) of Leu); 26.5, 26.0, 25.9, 25.5, 24.9, 24.3, 23.7, 21.5 (8q, 6Me of 3Aib and 2Me(5) of Leu). ESI-MS (NaI+MeOH): 804 (100, [M+Na]$^+$).

4.2.4. *Cyclo*(Gly^1-Aib^2-Leu^3-Aib^4-Phe^5-Aib^6) (1). Z-Gly-Aib-Leu-Aib-Phe-Aib-O*t*Bu (**5**) (0.555 g, 0.71 mmol) was *N*-deprotected according to GP A (H_2, 60 mg Pd/C, 10 ml MeOH, 20 h). Thus, 0.417 g (91%) of H-Gly-Aib-Leu-Aib-Phe-Aib-O*t*Bu were obtained as a white foam, which was

dissolved in abs. CH_2Cl_2 (20 ml), and TFA (20 ml) was added at rt. The mixture was stirred for 6 h. Excess TFA was removed under reduced pressure, followed by addition and evaporation of two portions of CH_2Cl_2 (10 ml). Upon drying under high vacuum, 0.461 g of the free linear hexapeptide were obtained as its TFA salt in quantitative yield.

HATU-mediated cyclization: 0.121 g (0.17 mmol) of H-Gly-Aib-Leu-Aib-Phe-Aib-OH·TFA were dissolved in abs. DMF (112 ml) and subjected to cyclization according to GP C, with HATU (0.194 g, 0.51 mmol), HOAt (69 mg, 0.51 mmol), and DIEA (1.2 ml). Reaction time: 1 day. Purification by CC (SiO_2, CH_2Cl_2/MeOH 10:1, EtOAc/MeOH 15:1) afforded 23 mg (24%) of pure cyclohexapeptide **1**.

DEPC-mediated cyclization: 0.121 g (0.17 mmol) of the free linear peptide TFA salt were dissolved in abs. DMF (112 ml) and the cyclization was performed according to GP D using DEPC (0.139 g, 0.85 mmol) and DIEA (1.2 ml). After 2 and 4 days of stirring, additional DEPC (69 mg, 0.425 mmol) was added. Reaction time: 6 days. The obtained yellow oil was purified by CC (SiO_2, EtOAc/MeOH 15:1, performed twice) to provide 51 mg (53%) of pure **1**.

PyAOP-mediated cyclization: 0.121 g (0.17 mmol) of the free linear peptide TFA salt were dissolved in abs. DMF (170 ml) and treated with PyAOP (0.441 g, 0.85 mmol), HOAt (0.116 g, 0.85 mmol), and DIEA (1.7 ml) following GP E. Purification by CC (SiO_2, CH_2Cl_2/MeOH 17:1, performed twice) afforded 30 mg (31%) of pure **1**. White powder. Mp (dec.) 284–286 °C. IR: 3317s, 3061w, 2968m, 2871w, 1704m, 1650s, 1536s, 1457m, 1390m, 1367m, 1264m, 1219m, 1188m, 1080w, 1029w, 744w, 698m. ^{1}H NMR: 8.22 (s, NH of Aib^2); 8.05 (s, NH of Aib^6); 7.80 (d, J=8.9 Hz, NH of Leu^3); 7.62 (d, J=7.6 Hz, NH of Phe^5); 7.55 (s, NH of Aib^4); 7.27–7.16 (m, 5 arom. H of Phe^5, NH of Gly^1); 4.33–4.27 (m, CH(2) of Leu^3 and CH(2) of Phe^5); 3.77 (dd, J=17.0, 5.8 Hz, 1H of CH_2(2) of Gly^1); 3.70 (dd, J=17.0, 3.2 Hz, 1H of CH_2(2) of Gly^1); 2.94 (dd, J=13.5, 7.7 Hz, 1H of CH_2(3) of Phe^5); 2.85 (dd, J=13.5, 7.2 Hz, 1H of CH_2(3) of Phe^5); 1.62–1.47 (m, CH_2(3) and CH(4) of Leu^3); 1.46, 1.38 (2s, 2Me of Aib^4); 1.37, 1.29 (2s, 2Me of Aib^2); 1.26, 1.19 (2s, 2Me of Aib^6); 0.88, 0.82 (2d, J=6.4 Hz, 2Me(5) of Leu^3). ^{13}C NMR: 174.2 (s, CO of Aib^4); 174.0 (s, CO of Aib^2); 173.7 (s, CO of Aib^6); 171.3 (s, CO of Leu^3); 170.0 (s, CO of Phe^5); 168.2 (s, CO of Gly^1); 137.7 (s, 1 arom. C of Phe^5); 129.3, 128.0, 126.2 (3d, 5 arom. CH of Phe^5); 56.4 (s, C(2) of Aib^4); 56.2 (s, C(2) of Aib^2); 56.0 (s, C(2) of Aib^6); 55.1 (d, C(2) of Phe^5); 50.9 (d, C(2) of Leu^3); 42.8 (t, C(2) of Gly^1); 40.2 (t, C(3) of Leu^3); 36.6 (t, C(3) of Phe^5); 27.0 (q, 1Me of Aib^6); 26.9 (q, 1Me of Aib^2); 25.9 (q, 1Me of Aib^4); 24.3 (d, C(4) of Leu^3); 23.47 (q, 1Me of Aib^2); 23.38 (q, Me(5) of Leu^3); 23.14 (q, 1Me of Aib^4); 23.09 (q, 1Me of Aib^6); 21.1 (q, Me(5) of Leu^3). ESI-MS (NaI+MeOH): 595 (100, $[M+Na]^+$). Anal. calcd for $C_{29}H_{44}N_6O_6$ (572.71): C 60.82, H 7.74, N 14.67; found: C 60.60, H 7.73, N 14.56.

4.3. Preparation of *cyclo*(Leu-Aib-Phe-Gly-Aib-Aib) (2)

4.3.1. *tert*-Butyl *N*-[(benzyloxy)carbonyl]-(*S*)-leucyl-dimethylglycyl-(*S*)-phenylalaninate (Z-Leu-Aib-Phe-O*t*Bu) (6). Z-Leu-Aib-OH[9] (0.25 g, 0.71 mmol) was coupled with HCl·H-Phe-O*t*Bu (0.202 g, 0.78 mmol) in abs. CH_2Cl_2/MeCN (6/2 ml) according to GP B, using PyBOP (0.371 g, 0.71 mmol) and DIEA (0.276 g, 2.14 mmol, overnight). Purification of the crude product by CC (SiO_2, CH_2Cl_2/MeOH 17:1, performed twice) afforded 0.362 g (92%) of tripeptide **6** as a white foam. IR: 3401m, 3368m, 3237m, 3033w, 2973m, 2951m, 1870w, 1720s, 1663s, 1651s, 1515s, 1457m, 1439m, 1389w, 1367m, 1246s, 1220m, 1166m, 1118w, 1040m, 861w, 846w, 780w, 755w, 743w, 700m. ^{1}H NMR: 8.05 (br s, NH of Aib); 7.52–7.48 (m, NH of Leu and NH of Phe); 7.33–7.17 (m, 10 arom. H); 5.06–4.95 (m, PhCH_2O), 4.34–4.31, 4.00–3.97 (2m, CH(2) of Phe and CH(2) of Leu); 2.96–2.93 (m, CH_2(3) of Phe); 1.70–1.31 (m, CH_2(3) and CH(4) of Leu, 2Me of Aib and Me_3C); 0.88–0.84 (m, 2Me(5) of Leu). ^{13}C NMR: 173.6, 171.7, 170.1 (3s, 3CO); 156.0 (s, CO (urethane)); 137.2, 136.8 (2s, 2 arom. C); 129.0, 128.1, 127.9, 127.6, 127.4, 126.3 (6d, 10 arom. CH); 80.5 (s, Me_3C); 65.2 (t, PhCH_2O); 55.8 (s, C(2) of Aib); 54.2, 53.3 (2d, C(2) of Leu and C(2) of Phe); 40.0, 36.8 (2t, C(3) of Leu and C(3) of Phe); 27.4 (q, Me_3C); 24.0 (d, C(4) of Leu); 25.5, 23.8, 22.8, 21.4 (4q, 2Me of Aib and 2Me(5) of Leu). ESI-MS (NaI+MeOH): 576 (100, $[M+Na]^+$). Anal. calcd for $C_{31}H_{43}N_3O_6$ (553.69): C 67.24, H 7.83, N 7.59; found: C 67.23, H 7.82, N 7.54.

4.3.2. Benzyl *N*-((*S*)-1-{[(1,1-dimethyl-2-{[1-(*S*)-benzyl-2-({2-[(1,1-dimethyl-2-{[1,1-dimethyl-2-(methylphenylamino)-2-oxoethyl]amino}-2-oxoethyl)amino]-2-oxoethyl}amino)-2-oxoethyl]amino}-2-oxoethyl)amino]oxomethyl}-3-methylbutyl) carbamate (Z-Leu-Aib-Phe-Gly-Aib-Aib-N(Me)Ph) (8). Z-Leu-Aib-Phe-O*t*Bu (**6**) (0.7 g, 1.26 mmol) was dissolved in CH_2Cl_2 (15 ml), TFA was added (15 ml), and the mixture was stirred for 6 h at rt. The solvent was then evaporated and the crude product filtered through a short SiO_2-column using CH_2Cl_2/MeOH (12:1) to give 0.586 g (93%) of Z-Leu-Aib-Phe-OH as a white foam, which was used directly in the next reaction.

Z-Gly-Aib-Aib-N(Me)Ph (**7**)[7] (0.234 g, 0.5 mmol) was *N*-deprotected following GP A (H_2, 25 mg Pd/C, 6 ml MeOH, overnight). The crude product was dried under vacuum to give 0.159 g (95%) of H-Gly-Aib-Aib-N(Me)Ph, which was used in the next reaction step without further purification.

The coupling of Z-Leu-Aib-Phe-OH (0.215 g, 0.43 mmol) with H-Gly-Aib-Aib-N(Me)Ph (0.159 g, 0.48 mmol) in abs. CH_2Cl_2 (6 ml) was achieved according to GP B, using PyAOP (0.26 g, 0.5 mmol) and DIEA (0.129 g, 1.0 mmol, overnight). Purification by CC (SiO_2, CH_2Cl_2/MeOH 20:1) afforded 0.21 g (60%) of hexapeptide **8** as a white foam. IR: 3309s, 3062w, 3032m, 2957m, 2872w, 1662s, 1594m, 1533s, 1455s, 1389m, 1364m, 1332m, 1266m, 1221m, 1173m, 1118m, 1091m, 1045w, 1028w, 922w, 741w, 704m. ^{1}H NMR (CD_3OD): 7.40–7.16 (m, 15 arom. H); 5.14–4.99 (m, PhCH_2O); 4.05–3.59 (m, CH(2) of Leu, CH(2) of Phe and CH_2(2) of Gly); 3.35–2.90 (m, MeN and CH_2(3) of Phe); 1.75–1.46 (m, CH_2(3) and CH(4) of Leu, 4Me of 2Aib); 1.30, 1.27 (2s, 2Me of Aib); 0.96–0.91 (m, 2Me(5) of Leu). ^{13}C NMR (CD_3OD): 177.0, 176.2, 176.1, 175.4,

174.4, 171.3 (6s, 6CO (amide)); 158.5 (s, CO (urethane)); 139.3, 138.1 (2s, 3 arom. C); 130.2, 130.1, 129.9, 129.4, 129.0, 128.5, 128.4, 128.2, 127.6 (9d, 15 arom. CH); 67.6 (t, Ph*C*H_2O); 58.6, 58.3, 57.7 (3s, 3 C(2) of 3Aib); 57.1, 55.3 (2d, C(2) of Leu and C(2) of Phe); 44.8, 41.3 (2t, C(3) of Leu and C(2) of Gly); 41.27 (q, MeN); 36.4 (t, C(3) of Phe); 25.7 (d, C(4) of Leu); 26.3, 25.3, 24.7, 23.2, 22.1 (5q, 6Me of 3Aib and 2Me(5) of Leu). ESI-MS (NaI+MeOH): 837 (100, $[M+Na]^+$). Anal. calcd for $C_{44}H_{59}N_7O_8 \cdot 1/3H_2O$ (819.99): C 64.45, H 7.33, N 11.96; found: C 64.35, H 7.36, N 11.96.

4.3.3. *cyclo*(Leu[1]-Aib[2]-Phe[3]-Gly[4]-Aib[5]-Aib[6]) (2). Peptide **8** (0.42 g, 0.52 mmol) was dissolved in MeCN (3 ml) and then 3 ml of 6 N HCl were added dropwise. The mixture was stirred at rt overnight. The MeCN was evaporated under reduced pressure and 2 N HCl (3 ml) was added. The product was extracted with CH_2Cl_2, the organic layer was dried (Na_2SO_4), filtered and concentrated under reduced pressure. After drying under vacuum, 0.369 g (98%) of Z-Leu-Aib-Phe-Gly-Aib-Aib-OH were obtained as a white foam. Then, 0.318 g (0.44 mmol) of this compound were *N*-deprotected according to GP A (H_2, 35 mg Pd/C, 6 ml MeOH, 20 h). After drying under vacuum, 0.241 g (93%) of the free linear hexapeptide were obtained as a pale yellow foam, which was used in the cyclization step without further purification.

HATU-mediated cyclization: 84 mg (0.14 mmol) of the free linear hexapeptide were dissolved in abs. DMF (200 ml) and subjected to macrolactamization according to GP C, with HATU (0.162 g, 0.43 mmol), HOAt (58 mg, 0.43 mmol), and DIEA (2 ml). Reaction time: 3 days. Purification by CC (SiO_2, CH_2Cl_2/MeOH 12:1, performed twice) afforded 13 mg (16%) of pure cyclohexapeptide **2** as a white foam.

DEPC-mediated cyclization: 88 mg (0.15 mmol) of the free linear precursor were dissolved in abs. DMF (100 ml) and the cyclization was performed according to GP D, using DEPC (0.141 g, 0.75 mmol) and DIEA (1 ml). After 2 and 4 days, additional DEPC (60.5 mg, 0.375 mmol) was added to the stirred mixture. Reaction time: 6 days. The obtained yellow oil was purified by CC (SiO_2, CH_2Cl_2/MeOH 14:1, performed trice) to afford 28 mg (33%) of pure **2** as a white foam.

PyAOP-mediated cyclization: 64 mg (0.11 mmol) of the free linear precursor were dissolved in abs. DMF (180 ml) and treated with PyAOP (0.169 g, 0.32 mmol), HOAt (44 mg, 0.32 mmol) and DIEA (1.8 ml) by following GP E. Purification by CC (SiO_2, CH_2Cl_2/MeOH 12:1, performed thrice) yielded 30 mg (48%) of pure **2** as a white foam. IR: 3327s, 3030w, 2957m, 2871w, 1657s, 1534s, 1469m, 1455m, 1385m, 1365m, 1277m, 1225m, 1179m, 1030w, 945w, 820w, 748w, 702m. ^{1}H NMR: 8.28 (s, NH of Aib[5]); 8.14 (s, NH of Aib[2]); 7.43 (br s, NH of Gly[4]); 7.41 (d, J=9.7 Hz, NH of Leu[1]); 7.39 (s, NH of Aib[6]); 7.32 (d, J=9.0 Hz, NH of Phe[3]); 7.24–7.14 (m, 5 arom. H of Phe[3]); 4.59–4.55 (m, CH(2) of Phe[3]); 4.28–4.20 (m, CH(2) of Leu[1] and 1H of CH_2(2) of Gly[4]); 3.62–3.59 (m, 1H of CH_2(2) of Gly[4]); 3.31–3.28 (m, 1H of CH_2(3) of Phe[3]); 2.93–2.89 (m, 1H of CH_2(3) of Phe[3]); 1.88–1.83 (m, 1H of CH_2(3) of Leu[1]); 1.72–1.68 (m, CH(4) of Leu[1]); 1.60–1.55 (m, 1H of CH_2(3) of Leu[1]); 1.43 (s, Me of Aib[6]); 1.31, 1.30 (2s, 2Me of Aib[5]); 1.23 (s, Me of Aib[6]); 1.12, 1.03 (2s, 2Me of Aib[2]); 0.899, 0.897 (2d, J=6.4, 6.8 Hz, 2Me(5) of Leu[1]). ^{13}C NMR: 173.5 (s, CO of Aib[2]); 173.2 (s, CO of Aib[5]); 173.0 (s, CO of Aib[6]); 172.9 (s, CO of Leu[1]); 170.7 (s, CO of Phe[3]); 169.1 (s, CO of Gly[4]); 138.6 (s, 1 arom. C of Phe[3]); 128.9, 127.9, 126.0 (3d, 5 arom. CH of Phe[3]); 56.9 (s, C(2) of Aib[6]); 56.3 (s, C(2) of Aib[5]); 55.9 (s, C(2) of Aib[2]); 53.2 (d, C(2) of Phe[3]); 50.1 (d, C(2) of Leu[1]); 41.2 (t, C(2) of Gly[4]); 40.4 (t, C(3) of Leu[1]); 35.5 (t, C(3) of Phe[3]); 28.0 (q, Me of Aib[6]); 26.1 (q, Me of Aib[5]); 25.7 (q, Me of Aib[2]); 24.0 (d, C(4) of Leu[1]); 23.8 (q, Me(5) of Leu[1]); 23.7 (q, Me of Aib[2]); 23.22 (q, Me of Aib[6]); 23.19 (q, Me of Aib[5]); 20.9 (q, Me(5) of Leu[1]). ESI-MS (NaI+MeOH): 595 (100, $[M+Na]^+$). Anal. calcd for $C_{29}H_{44}N_6O_6 \cdot 1/2H_2O$ (581.71): C 59.88, H 7.80, N 14.45; found: C 60.02, H 7.98, N 14.36.

4.4. X-Ray crystal-structure determination of 1 and 2

All measurements were made on a Nonius KappaCCD area-detector diffractometer[38] using graphite-monochromated MoK_α radiation (λ 0.71073 Å) and an Oxford Cryosystems Cryostream 700 cooler. The data collection and refinement parameters are given below[39] and views of the molecules are shown in Figures 2 and 3. The intensities were corrected for Lorentz and polarization effects, but not for absorption. Standard reflection intensities were not monitored. Equivalent reflections, other than Friedel pairs, were merged. The structures were solved by direct methods using SIR92,[40] which revealed the positions of all non-hydrogen atoms.

The *iso*-propyl part of the Leu side chain in **1** is disordered. Two positions were defined for the disordered atoms and refinement of the site occupation factors yielded a value of 0.53(2) for the major conformation. Bond length and similarity restraints were applied to all chemically equivalent bond lengths and angles involving the disordered atoms. Neighboring atoms within and between each conformation of the disordered isopropyl group were also restrained to have similar atomic displacement parameters.

The asymmetric unit of **2** contains one molecule of the peptide plus two water molecules, one disordered EtOH molecule and one disordered *i*-PrOH molecule. Two positions were defined for each of the atoms of the two disordered solvent molecules and the site occupation factors of the major conformations refined to 0.51(2) and 0.50(2) for the EtOH and *i*-PrOH molecules, respectively. Similarity restraints were applied to the chemically equivalent bond lengths within the disordered molecules and neighboring atoms within and between each conformation of the disordered molecule were also restrained to have similar atomic displacement parameters.

The non-hydrogen atoms were refined anisotropically. The amide H-atoms in both structures, and the water H-atoms in **2**, were placed in positions indicated by a difference electron density map and their positions were allowed to refine together with individual isotropic displacement parameters. All remaining H-atoms were placed in geometrically calculated positions and refined using a riding model where each H-atom was assigned a fixed isotropic

displacement parameter with a value equal to $1.2U_{eq}$ of its parent C-atom ($1.5U_{eq}$ for the methyl groups). The orientations of the hydroxy O–H vectors in the solvent molecules of **2** were chosen so as to be directed towards the nearest hydrogen bond acceptor atom. The refinement of each structure was carried out on F^2 using full-matrix least-squares procedures, which minimised the function $\Sigma w(F_o^2-F_c^2)^2$. Corrections for secondary extinction were applied. For **1** and **2**, 11 and two reflections, respectively, were omitted from the final refinement. In each case, the enantiomer used in the refinement was chosen to correspond with the known *S*-configuration of the chiral centers derived from precursor molecules.

Neutral atom scattering factors for non-hydrogen atoms were taken from Ref. 41 and the scattering factors for H-atoms were taken from Ref. 42. Anomalous dispersion effects were included in F_c;[43] the values for f' and f'' were those of Ref. 44. The values of the mass attenuation coefficients are those of Ref. 45. All calculations were performed using the *SHELXL97* program.[46]

In **1**, each N–H group of the peptide molecule acts as a donor for hydrogen bonds. Two of the interactions, N(1)–H and N(10)–H, are intramolecular hydrogen bonds. N(1)–H interacts with the amide O(12)-atom that is diagonally opposed in the peptide ring to give a loop with a graph set motif[47] of S(10). N(10)–H does not interact with a diametrically opposed amide O-atom, but forms bifurcated intramolecular hydrogen bonds with the amide O-atoms (O(6) and O(12), respectively) of the two adjacent peptide units. These two interactions have graph set motifs of S(7) and S(5). N(4)–H forms an intermolecular hydrogen bond with the amide O-atom of the same peptide unit of a neighboring molecule and thereby links the molecules into extended chains which run parallel to the [010] direction and have a graph set motif of C(4). N(7)–H, N(13)–H and N(16)–H form intermolecular hydrogen bonds with amide O-atoms of almost diagonally opposed peptide units from three different neighboring molecules. Each of these interactions links the molecules into extended chains which run parallel to the [010] direction and have a graph set motif of C(10). Together, the intermolecular hydrogen bonds link the molecules into extended two-dimensional networks which lie parallel to the (001) plane.

In **2**, all available N–H and O–H donors in the structure are involved in hydrogen bonds. The peptide molecule has two intramolecular hydrogen N–H…O bonds which diagonally cross the molecule to link the amide N–H donors with amide O-atoms that are seven atoms further along the peptide backbone. Each of these interactions has a graph set motif of S(10), which, despite the cyclic nature of the peptide, is the same as usually observed in open chain peptides. The remaining four amide N–H donors form intermolecular hydrogen bonds with the O-atoms from each of the four symmetry-independent solvent molecules, so that the two water molecules, the EtOH molecule and the *i*-PrOH molecule each accept one hydrogen bond. Each of the solvent O–H donors, in turn, forms an intermolecular hydrogen bond with an amide O-atom of a peptide molecule. The EtOH and *i*-PrOH molecules both act as acceptors and donors of hydrogen bonds involving the same peptide molecule to give a closed trimeric system. In each case, this builds a loop with a graph set motif of $R_2^2(10)$. In contrast, the water molecules form hydrogen bonds between different peptide molecules and thereby link all of the peptide and solvent molecules in the structure into an infinite three-dimensional framework. Although there are two symmetry-independent water molecules in the structure, each generates the same hydrogen-bonding pattern. The path via one H-atom from each water molecule creates a chain with a binary graph set motif of $C_2^2(7)$, while the path via the other H-atom from each water molecule creates a chain with a binary graph set motif of $C_2^2(10)$.

Crystal data for **1**: $C_{29}H_{44}N_6O_6$, $M=572.70$, colorless, prism, crystal dimensions $0.10\times0.12\times0.25$ mm, orthorhombic, space group $P2_12_12_1$, $Z=4$, reflections for cell determination 3164, 2θ range for cell determination 4–50°, $a=9.7189(2)$ Å, $b=10.0614(2)$ Å, $c=31.9151(7)$ Å, $V=3120.8(1)$ Å^3, $T=160$ K, $D_X=1.219$ g cm^{-3}, $\mu(\mathrm{Mo}K_\alpha)=0.0863$ mm^{-1}, $2\theta_{(max)}=50°$, total reflections measured 27,778, symmetry independent reflections 3146, reflections with $I>2\sigma(I)$ 2265, reflections used in refinement 3135, parameters refined 433; restraints 68, $R(F)$ [$I>2\sigma(I)$ reflections] = 0.0407, $wR(F^2)$ [all data] = 0.0870 ($w=[\sigma^2(F_o^2)+(0.0332P)^2]^{-1}$, where $P=(F_o^2+2F_c^2)/3$), goodness of fit 1.000, secondary extinction coefficient 0.005(1), final Δ_{max}/σ 0.001, $\Delta\rho$ (max; min) = 0.18; −0.18e Å^{-3}.

Crystal data for **2**: $C_{29}H_{44}N_6O_6\cdot$EtOH$\cdot$*i*-PrOH$\cdot2H_2O$, $M=714.89$, colorless, prism, crystal dimensions $0.30\times0.30\times0.35$ mm, monoclinic, space group $P2_1$, $Z=2$, reflections for cell determination 4747, 2θ range for cell determination 4–55°, $a=10.0827(1)$ Å, $b=12.5382(1)$ Å, $c=15.7976(2)$ Å, $\beta=96.4866(4)°$, $V=1984.33(4)$ Å^3, $T=160$ K, $D_X=1.196$ g cm^{-3}, $\mu(\mathrm{Mo}K_\alpha)=0.0878$ mm^{-1}, $2\theta_{(max)}=55°$, total reflections measured 44,105, symmetry independent reflections 4757, reflections with $I>2\sigma(I)$ 4044, reflections used in refinement 4755, parameters refined 573; restraints 164, $R(F)$ [$I>2\sigma(I)$ reflections] = 0.0459, $wR(F^2)$ [all data] = 0.1280 ($w=[\sigma^2(F_o^2)+(0.0817P)^2+0.2355P]^{-1}$, where $P=(F_o^2+2F_c^2)/3$), goodness of fit 1.040, secondary extinction coefficient 0.025(4), final Δ_{max}/σ 0.001, $\Delta\rho$ (max; min) = 0.42; −0.29e Å^{-3}.

Acknowledgements

We thank the analytical sections of our institute for spectra and analyses. Financial support of the *Swiss National Science Foundation* and *F. Hoffmann-La Roche AG*, Basel, is gratefully acknowledged.

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