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Synthesis and conformational preferences of unnatural tetrapeptides containing L-valine units

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Abstract—The stereoselective synthesis of non-proteinogenic tetrapeptides **7**, **16** and **17** containing two L-valine units and two modified α-amino acids (proline and aspartic acid) has been accomplished starting from the L-valine derived chiral synthon **1**. Investigations of the conformational preference and structure of these unnatural peptides were carried out using ^{1}H NMR and IR spectroscopic techniques and a conformational analysis based on quenched molecular dynamics (QMD).

1. Introduction

In a previous paper,[1] we reported the stereoselective synthesis of the unusual di- and tripeptides containing the L-valine unit and a cyclic unnatural α-aminoacid. The importance of replacing natural amino acids in peptides with non-proteinogenic counterparts, in order to obtain drug-like target molecules, has stimulated our interest to undertake the synthesis of new and more complex unnatural peptides. In particular, we are interested in the synthesis of peptidomimetic structures because they exhibit therapeutic effects which are similar to those of natural peptides but with the advantage of metabolic stability. The strategy followed to accomplish the asymmetric synthesis of the title pseudotetrapeptide is identical to that previously acquired on the stereoselective approach to unnatural peptides containing the L-valine and proline[1] or aspartic acid derivatives.[2] In this approach we started from a chiral monolactim ether, easily obtained from L-valine.

The non-proteinogenic peptides **6**, **7**, **14**, **15**, **16** and **17** (see Schemes 1 and 2) have been examined using spectroscopic techniques (^{1}H NMR and IR) to understand if the cyclic unnatural α-aminoacid (a proline derivative) behaves as a scaffold, promoting a compact conformation and the reverse turn formation through intra-molecular hydrogen bonds, that is, a U-shaped structure. Furthermore, to analyze the conformational features of these peptides, a theoretical investigation based on a 'quenched molecular dynamics' (QMD) approach, has been carried out.

2. Results and discussion

The stereoselective synthesis followed, makes use of chiral synthon **2** obtained from (5*S*)-4-benzyl-6-ethoxy-5-isopropyl-3-oxo-2,3,4,5-tetrahydro-pyrazine **1**, a monolactim ether easily synthesized from L-valine, as described in our previous papers.[3] Chiral sinthon **2**, a masked unnatural cyclic dipeptide (constituted by L-valine and an aspartic acid derivative), was reacted with another unnatural dipeptide **3**[1,2] (containing L-valine and a proline derivative) to obtain the more complex structure **4**. After alkaline hydrolysis, acid derivative **5** was converted into amide **6** by treating with cyclohexylamine in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM), obtained starting from 2,4-dimethoxy-6-chloro-1,3,5-triazine.[4]

The acid hydrolysis under mild conditions of intermediate **6** and subsequent acylation with acetyl chloride allowed us to obtain tetrapseudopeptide **7**, containing L-valine (red), modified proline (green) and modified aspartic acid (blue), in a satisfactory overall yield.

In order to synthesize tetrapseudopeptides **16** and **17**, which differ from **7** by the substituent and the configuration of the proline derivative, we employed pseudopeptides **8** or **9** already synthesized by us.[1] In fact, chiral synthon **2** was

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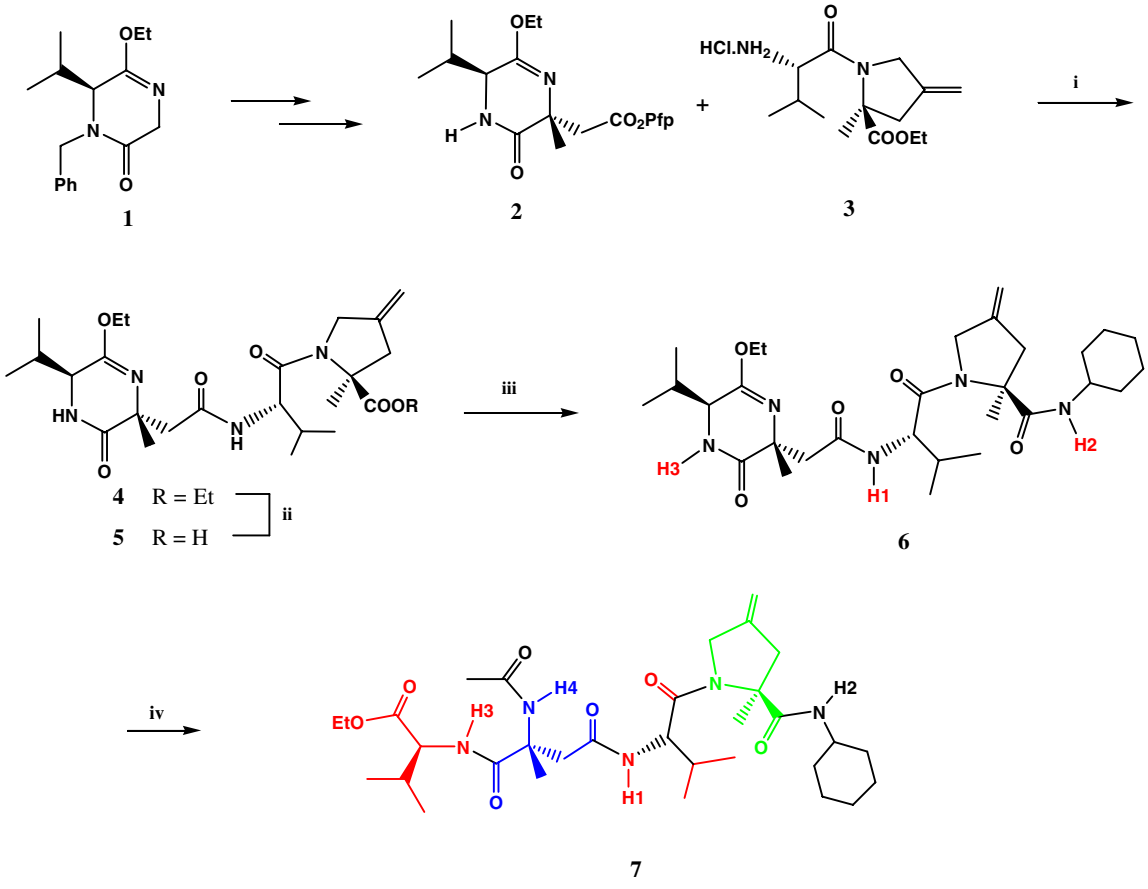
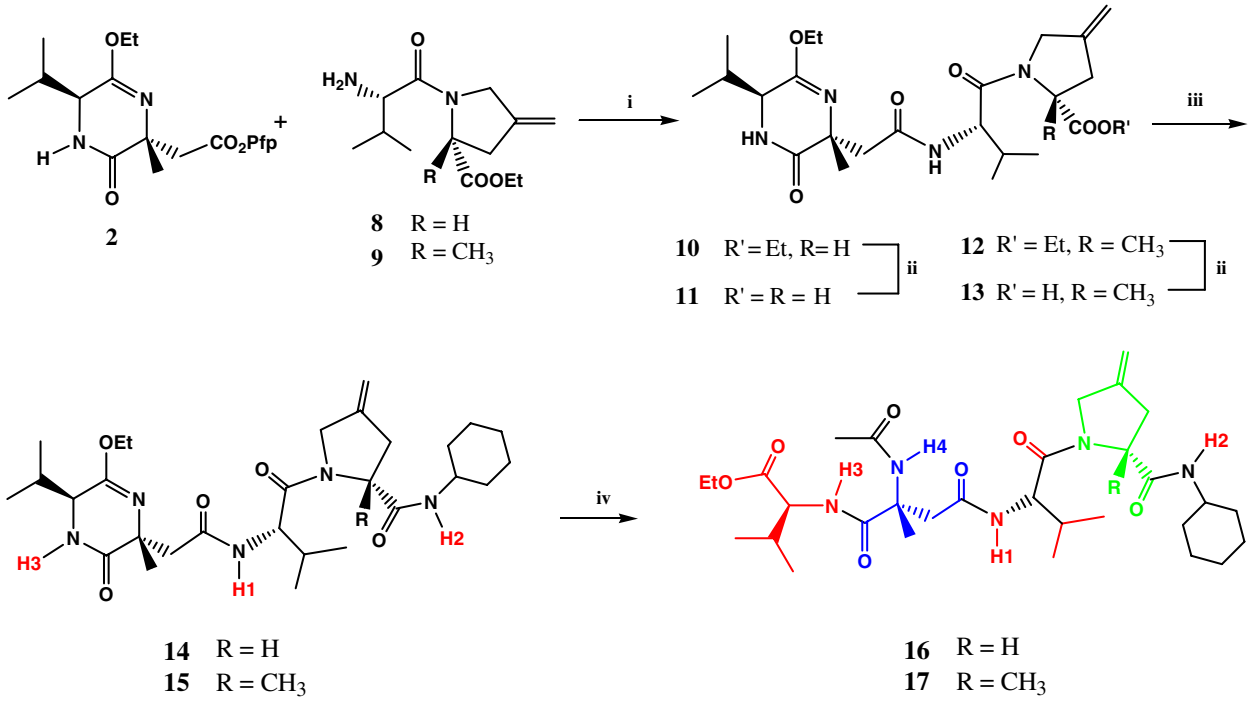


Scheme 1. Reagents and conditions: (i) CH_2Cl_2 in the presence of Et_3N at rt; (ii) 2 M NaOH in EtOH; (iii) cyclohexylamine in THF in the presence of DMTMM at rt; (iv) 0.5 M HCl, then CH_3COCl in CH_2Cl_2/Et_3N.



Scheme 2. Reagents and conditions: (i) in CH_2Cl_2 in the presence of Et_3N, at rt; (ii) 2 M NaOH in EtOH; (iii) cyclohexylamine in THF in the presence of DMTMM at rt; (iv) 0.5 M HCl, then CH_3COCl in CH_2Cl_2/Et_3N.

matched with nucleophiles **8** or **9** giving compounds **10** or **12**, respectively. These intermediates, submitted to the reactions sequence above described to obtain compound **7**, gave tetrapseudopeptides **16** and **17** in satisfactory overall yields (Scheme 2).

3. ^{1}H NMR and IR studies

In order to determine the structural features of tetrapseudopeptides **7**, **16**, **17** and their precursors **6**, **14**, **15**, spectroscopic studies using ^{1}H NMR and IR techniques were carried out.[1,5,6] Information on the existence of the hydrogen bonds was obtained from the chemical shift of the amide NH bond, the value of the temperature coefficient ($\Delta\delta_{NH}/\Delta T$) and the δ_{NH} change after addition of DMSO, which is a strongly competitive solvent in hydrogen bond formation. Further information can be attained from the infrared stretching absorption of the amide NH bond. When the spectra are obtained in dilute solutions, a broad band in the range 3300–3350 cm^{-1} indicates the presence of an intra-molecular hydrogen bond, while a sharp band higher than 3400 cm^{-1} can be assigned to a free amide NH bond. In Table 1, we have collected the meaningful ^{1}H NMR and IR data of the various substrates investigated in diluted solutions. For clarity their amide protons are labelled as **H1**, **H2**, **H3** and **H4** (see Schemes 1 and 2). The ^{1}H NMR spectra assignments of the **H1**, **H2**, **H3** and **H4** protons (which are not magnetically equivalent) were achieved on the basis of the signal multiplicity as well as by irradiation and/or NOE measurements.

Table 1. Meaningful ^{1}H NMR and IR data for substrates **6**, **7**, **14**, **15**, **16** and **17**

	δ_{NH} (ppm) (2 mM $CDCl_3$)	δ_{NH} (ppm) (2 mM $CDCl_3$/DMSO 4:1)	$\lvert\Delta\delta_{NH}/\Delta T\rvert$ (ppb/°C) (in $CDCl_3$)	IR (cm^{-1}) (2 mM $CHCl_3$)
6[a]	**H1** 6.58	**H1** 7.47	**H1** 0.6	3376, 3394, 3420
	H2 6.41	**H2** 6.65	**H2** 1.4	
	H3 5.70	**H3** 7.01	**H3** 1.6	
7[a]	**H1** 6.91	**H1** 7.91	**H1** 5.5	3290, 3378, 3419
	H2 6.48	**H2** 6.64	**H2** 0.5	
	H3 8.04	**H3** 7.85	**H3** 2.6	
	H4 7.35	**H4** 7.72	**H4** 2.6	
14	**H1** 7.20	**H1** 7.90	**H1** 6.4	3345, 3400
	H2 6.64	**H2** 6.95	**H2** 2.6	
	H3 5.91	**H3** 7.14	**H3** 2.6	
15	**H1** 7.27	**H1** 8.00	**H1** 6.0	3290, 3366, 3400
	H2 6.43	**H2** 6.52	**H2** 2.0	
	H3 5.80	**H3** 7.20	**H3** 1.1	
16	**H1** 7.13	**H1** 7.43	**H1** 5.0	3341, 3426
	H2 6.75	**H2** 6.96	**H2** 2.0	
	H3 7.87	**H3** 7.94	**H3** 0.4	
	H4 7.06	**H4** 7.77	**H4** 0.4	
17	**H1** 6.97	**H1** 8.10	**H1** 2.0	3294, 3363, 3426
	H2 6.58	**H2** 6.58	**H2** 1.4	
	H3 7.96	**H3** 7.80	**H3** 0.9	
	H4 7.30	**H4** 7.60	**H4**[b]	

[a] δ_{NH} Values of the more abundant conformer (see Section 6).

[b] It was not possible to determine the temperature coefficient, $\Delta\delta_{NH}/\Delta T$, because in the range of temperatures from 30 to 50 °C, the signal of **H4** is hidden by the $CHCl_3$ present in $CDCl_3$.

From the spectroscopic data reported in Table 1, it can be deduced that in pseudopeptides **6** and **17**, the **H1** protons most probably do not form hydrogen bonds because their chemical shifts are <7 ppm and, more importantly, the signals are characterized by a significant downfield shift (0.89–1.13 ppm) upon addition of 20% DMSO. Furthermore, the IR spectra show sharp bands at $\nu > 3400$ cm^{-1}, that is, in the characteristic region of free NH amide absorbance. In our opinion also in **7**, the **H1** proton, in spite of the large temperature coefficient (5.5 ppb), does not form hydrogen bonds. This because a remarkable downfield shift (1 ppm) upon addition of 20% DMSO and an IR band at $\nu = 3419$ cm^{-1} were observed. The δ_{NH} values of **H1** in **14** and **15** in connection with the downfield shift upon addition of 20% DMSO ($\Delta\delta_{NH} = 1.0$, 0.7, 0.73 ppm) and the absolute values of temperature coefficients (5.5, 6.4, 6.0 ppb/°C) are indicative of the existence of a dynamic equilibrium between hydrogen-bonded and a non-hydrogen-bonded structure. On the basis of the chemical shift value, the downfield shift upon addition of 20% DMSO and the absolute value of the temperature coefficient (5 ppb/°C), we believed that also in **16**, the **H1** proton is involved in a dynamic equilibrium between a hydrogen-bonded and a non-hydrogen-bonded structure. However, the moderate downfield shift upon addition of 20% DMSO (0.3 ppm), suggests that **H1** in **16** is probably involved in a hydrogen bond, which is stronger than in **14** and **15**.

It is important to stress that the δ_{NH} value below 7 ppm, registered in all substrates for the **H2** proton, is almost certainly due to the shielding effect induced by the proximal cyclohexane ring. This aspect and the moderate downfield shift upon addition of 20% DMSO, suggest that in all these substrates, the **H2** proton is involved in an intramolecular hydrogen bond, in spite of the chemical shift value being below 7 ppm, as generally reported in the literature.[6]

The spectroscopic data indicate that in **6**, **14** and **15**, the **H3** proton does not participate with intra-molecular hydrogen bonds. Conversely, in pseudotetrapeptides **7**, **16** and **17** we can assert that both **H3** and **H4** are involved in hydrogen bond formation. However, the hydrogen bond involving **H4** is presumably weaker in **16** with respect to **7** and **17**, owing to the larger downfield variation of the chemical shift δ_{NH_4} observed for these two compounds after the addition of 20% DMSO (see Table 1).

4. Molecular modelling and conformational analysis

To explore the conformational space of pseudopeptides **6**, **7**, **14**, **15**, **16** and **17** and obtain useful information for understanding spectroscopic data, we carried out a high-temperature QMD. The QMD protocol, which has been used over the last decade to produce reasonable molecular structure of a variety of peptides,[7,8] generates representations of families of low-energy conformational structures

and provides statistical indications about selected hydrogen bonds.

Representative conformations of the six peptides, obtained from the cluster analysis, are depicted in Figures 1–3. These conformations correspond to the most populated structures within different sets of clusters. These sets have been obtained by grouping together the original clusters on the basis of the similarity of the hydrogen bond pattern (see Computational details in Section 6). A list of hydrogen bond lifetimes for each compound is given in Table 2.

Inspection of H-bond lifetimes and cluster analysis indicate **6** and **7** as the most stable structures (see Fig. 1) since they hold their β-turn motif during the whole molecular dynamics. In structure **6**, the O4···**H2**(N) hydrogen bond has a lifetime of 98.55% and is responsible for the stability of the 10-membered cycle of the β-turn. The same hydrogen bond plays a key-role in structure **7** to maintain the cyclic skeleton. In this case the O4···**H2**(N) hydrogen bond has a lifetime of 96.20%.

Furthermore, in structure **6** and **7** **H1** is involved in the formation of hydrogen bonds with a rather short lifetime. In **6**, **H1** interacts with O7 and N5 (lifetimes of 21.35% and 18.10%, respectively), while in **7** this proton forms a hydrogen bond with O6 (lifetime of 18.10%). Additional hydrogen bonds involving **H3** (O5···**H3**(N), lifetime of 94.45%) and **H4** (O4···**H4**(N), lifetime of 67.05%) are observed in compound **7**. These computational results are in rather good agreement with the ^{1}H NMR data which indicate the presence of hydrogen bonds involving **H2** in compound **6** and hydrogen bonds involving **H2**, **H3** and **H4** for compound **7** (see the previous section for the discussion of the data reported in Table 1).

The cluster analysis shows that compound **14** can exist in many different conformations that are characterized by similar populations (some representative conformations are reported in Fig. 2). This species is characterized by a rather undefined helicoidal structure. The lifetimes of the hydrogen bonds (never larger than 40%) are in agreement with this unfolded structure. Only **H1** and **H2** and not **H3** seem to be involved in hydrogen bonds in agreement with the indications of ^{1}H NMR data.

The addition of a methyl group in the five-membered ring (compound **15**) causes the breaking of the helicoidal structure and the pseudopeptide adopts a β-turn structure. In this case, two dominant conformations (see Fig. 2) can be identified from the cluster analysis. In both these conformations, the O4···**H2**(N) hydrogen bond can be recognized (lifetime of 77.65%). This interaction is responsible for the stability of the 10-membered cyclic structure of the β-turn. In only one of these two representative conformations, the **H1** proton is engaged in a hydrogen bond (N5···**H1**(N)) with a lifetime of 29.30%. This computational evidence is consistent with the ^{1}H NMR results that clearly indicate the existence of a hydrogen bond involving **H2**, the existence of less populated conformations characterized by a **H1** hydrogen bond and the absence of hydrogen bonds involving **H3**.

Pseudopeptide **16** is an interesting example of a γ-turn structure. In the most populated conformation (see Fig. 3), hydrogen bonds involving **H4** (O4···**H4**(N), life-

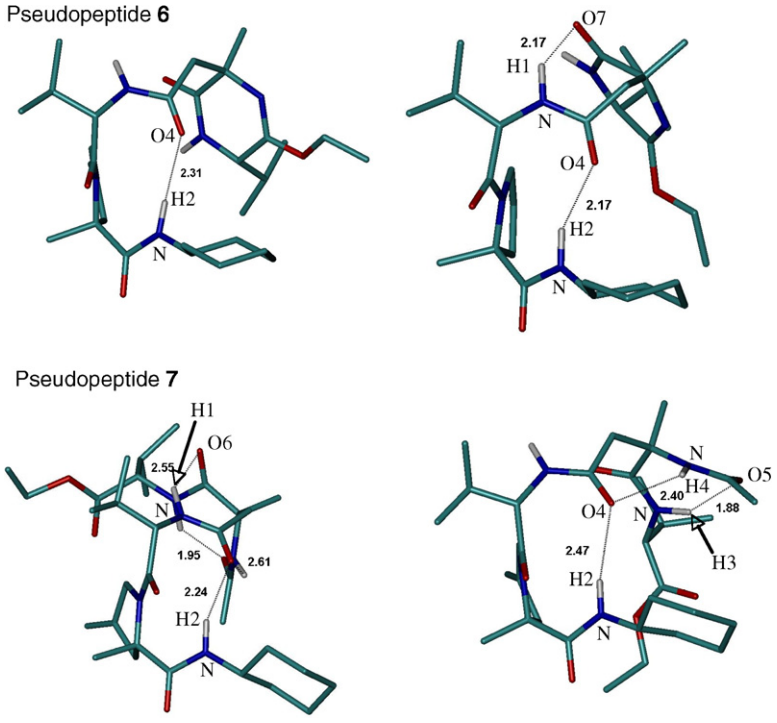


Figure 1. Representative conformations of pseudopeptides **6** and **7** (atom distances are in Å).

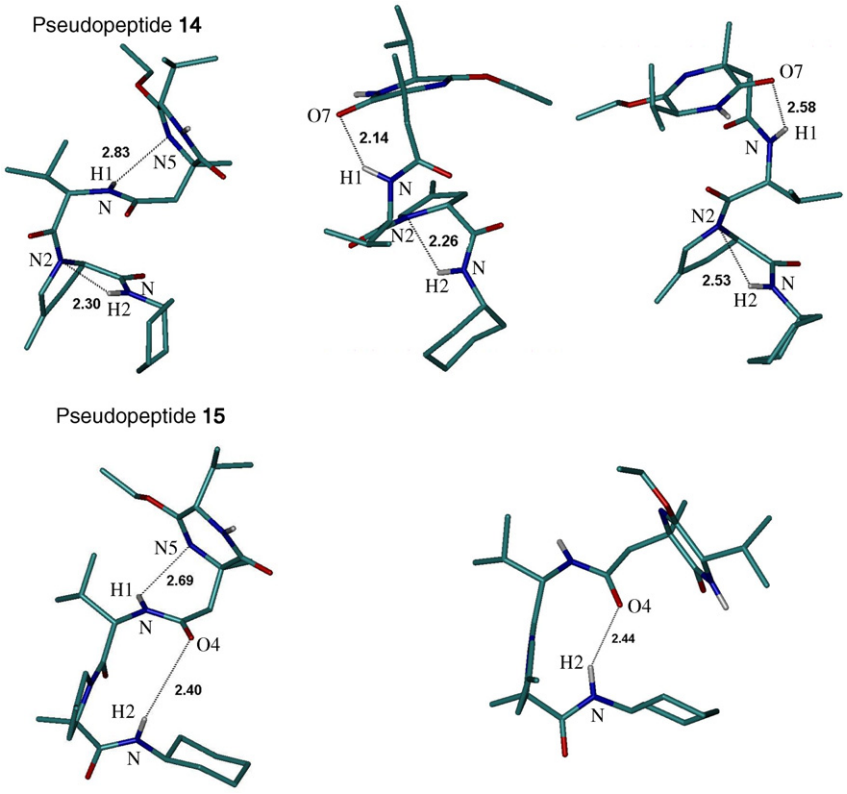


Figure 2. Representative conformations of pseudopeptides **14** and **15** (atom distances are in Å).

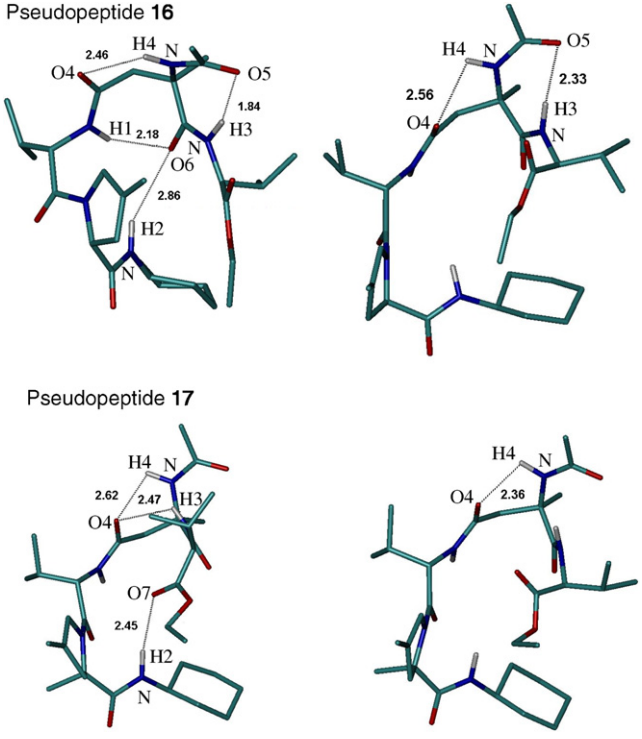


Figure 3. Representative conformations of pseudopeptides **16** and **17** (atom distances are in Å).

time of 80.25%), **H3** (O5···**H3**(N), lifetime of 85.15%), **H1** (O6···**H1**(N), lifetime of 57.10%) and **H2** (O6···**H2**(N), lifetime of 26.75%) are evident. The **H4** and **H3** hydrogen bonds are also present in the second representative conformation of Figure 3. Also in this case the conformational modelling is in agreement with the ^{1}H NMR data indicating that all four protons form hydrogen bonds.

Pseudopeptide **17**, obtained by the addition of a methyl group to the five-membered ring in **16**, is characterized by the equilibrium between two main conformations (see Fig. 3). While the O7···**H2**(N) interaction can be recognized only in the former one, the O4···**H4**(N) hydrogen bond is present in both conformations. However, cluster analysis shows that **H2** is engaged in other hydrogen bonds with lifetimes approximately in the range 33–7%. This is in agreement with the ^{1}H NMR results that indicate the existence of hydrogen bonds involving **H2** and **H4**.

5. Conclusions

Herein we have achieved a new synthetic approach for obtaining optically active tetrapseudopeptides. The synthesis of new peptidomimetic structures was performed in a satisfactory overall yield starting from chiral synthon **1**, a monolactim ether easily synthesized from L-valine. This approach represents a new and simple synthetic path for obtaining tetrapseudopeptides containing two L-valine residues, one modified proline unit and one modified aspartic acid unit.

Spectroscopic investigation using ^{1}H NMR and IR techniques combined with conformational analysis based on

Table 2. Lifetimes for the various hydrogen bonds in substrates **6**, **7**, **14**, **15**, **16** and **17**

Structure	Hydrogen bond	Lifetime (%)
6	O4–H2	98.55
	N2–H2	33.85
	O7–H1	21.35
	O3–H2	15.40
	N5–H1	8.30
7	O4–H2	96.20
	O5–H3	94.45
	O4–H4	67.05
	N2–H2	33.35
	O6–H1	18.10
	O3–H2	16.90
14	N2–H2	39.30
	N5–H1	30.25
	O7–H1	27.00
	O3–H2	19.65
	O1–H1	10.50
	O6–H1	6.58
15	O4–H2	77.65
	N2–H2	38.70
	N5–H1	29.30
	O3–H2	25.60
	O6–H1	8.30
16	O5–H3	85.15
	O4–H4	80.25
	O6–H1	57.10
	N2–H2	34.90
	O6–H2	26.75
	O3–H2	19.50
17	O4–H4	48.20
	O5–H3	36.90
	N2–H2	33.25
	O7–H2	33.05
	O4–H3	31.75
	O3–H2	26.00
	O8–H2	14.60
	O4–H2	7.10
	O6–H1	5.95

quenched molecular dynamics (QMD) have been demonstrated to be a valid tool to elucidate the structures of the various tetrapeptides and to demonstrate the existence in all cases of intra-molecular hydrogen bonds involving carbonyl oxygens and amide protons.

It is worth noting that a value of δ_{NH}, larger or smaller than 7, is not enough to indicate the presence or absence of hydrogen bond interactions, respectively. This is due to the fact that in complex structures, such as those examined here, shielding or deshielding effects of neighboring groups can play an important role, as evidenced by the signals of the **H2** protons in all substrates. To decide about the existence of hydrogen bonds, other factors (δ_{NH} change upon addition of strongly competitive solvents such as DMSO, temperature coefficient and IR stretching of amidic N–H) must be taken into account.

6. Experimental

6.1. General information

^{1}H and ^{13}C NMR spectra were recorded on a Gemini spectrometer at 300 MHz (in about 15 mM solutions) using $CDCl_3$ as solvent, unless otherwise stated. Chemical shifts are reported in ppm relative to $CDCl_3$ while the coupling constants (J) are in Hz. IR spectra were recorded on a Nicolet FT 380 spectrophotometer. Optical rotation values were measured at 25 °C on a Perkin–Elmer 343 polarimeter. Dry THF was distilled from sodium benzophenone ketyl. Chromatographic separations were performed with silica gel 60 (230–400 mesh).

The synthesis and spectroscopic data of compounds **1**, **3**, **8** and **9** are reported in Ref. 3 while in Ref. 2 the data for compound **2** are reported.

6.1.1. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino]-3-methyl-butyryl}-2(*S*)-methyl-4-methylene-pyrrolidine-2-carboxylic acid ethyl ester, 4. The activated ester **2** (1.8 g, 4.3 mmol) was added to a solution of **3** (1.3 g, 4.3 mmol) and triethylamine (0.9 mL, 6.43 mmol) in dry CH_2Cl_2 (10 mL) under an inert atmosphere. The reaction mixture was stirred at rt for about 24 h, then the organic phase, after a rapid washing with 0.1 M HCl, was dried over $CaCl_2$ and evaporated under vacuum. The residue was submitted to purification by silica gel chromatography eluting with hexane/ethyl acetate and the pure product was recovered as an oil in an 82% yield. ^{1}H NMR δ: 0.88 (d, 3H, $J = 6.9$); 0.92 (d, 3H, $J = 6.9$); 0.99 (m, 6H); 1.26 (t, 3H, $J = 7$); 1.45 (s, 3H); 1.49 (s, 3H); 2.0 (m, 1H); 2.3 (m, 1H); 2.46 (d, 1H, $J = 14.4$); 2.52 (d, 1H, $J = 14.6$); 2.89 (d, 1H, $J = 14.4$); 3.07 (d, 1H, $J = 14.6$); 4.0–4.25 (m, 6H); 4.37 (m, 2H); 4.47 (dd, 1H, $J = 7.4$, 9.2); 5.07 (m, 2H); 5.73 (s, 1H); 6.61 (d, 1H, $J = 9$). ^{13}C NMR δ: 13.9, 14.0, 16.2, 17.7, 18.4, 19.0, 20.5, 28.3, 30.7, 31.1, 44.8, 47.2, 51.7, 55.2, 58.6, 58.7, 61.1, 61.3, 65.9, 109.0, 141.5, 157.3, 169.9, 170.0, 172.7, 173.6. $[\alpha]_D = -31.3$ (c 1.4, $CHCl_3$). Anal. Calcd for $C_{26}H_{42}N_4O_6$: C, 61.64; H, 8.36; N, 11.06. Found: C, 61.80; H, 8.34; N, 11.02.

6.1.2. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino]-3-methyl-butyryl}-2(*S*)-methyl-4-methylene-pyrrolidine-2-carboxylic acid, 5. 2 M NaOH (0.6 mL) was added to a solution of **4** (0.2 g, 0.4 mmol) in ethanol (3 mL) and the reaction was stirred at room temperature and monitored by TLC. After about 3 h, ethanol was evaporated in vacuo and CH_2Cl_2 was added. The organic solution was rapidly washed with 0.5 M HCl dried over $CaCl_2$ and then evaporated to dryness under vacuum. The oily residue was pure to the HPLC analysis. ^{1}H NMR δ: 0.88 (d, 3H, $J = 6.8$); 0.9 (d, 3H, $J = 6.8$); 0.97 (d, 3H, $J = 6.8$); 1.03 (s, 3H, $J = 6.8$); 1.27 (t, 3H, $J = 7$); 1.47 (s, 3H); 1.51 (s, 3H); 2.0 (m, 1H); 2.51 (d, 1H, $J = 14.4$); 2.61 (d, 1H, $J = 14.4$); 3.04 (d, 2H, $J = 14.4$); 4.0–4.6 (m, 6H); 5.1 (m, 2H); 7.02 (d, 1H, $J = 8.8$); 7.19 (s, 1H). ^{13}C NMR δ: 14.0, 16.1, 17.9, 18.3, 18.9, 20.5, 28.5, 30.6, 30.9, 44.9, 47.0, 51.9, 55.4, 58.4, 58.6, 61.3, 66.4, 108.8, 141.7, 157.3, 170.0, 170.2, 174.8,

176.4. $[\alpha]_D = -41.5$ (c 1.0, $CHCl_3$). Anal. Calcd for $C_{24}H_{38}N_4O_6$: C, 60.23; H, 8.0; N, 11.71. Found: C, 59.98; H, 8.03; N, 11.74.

6.1.3. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino}-3-methyl-butyryl}-2(*S*)-methyl-4-methylene-pyrrolidine-2-carboxylic acid cyclohexyl amide, 6. Cyclohexylamine (0.1 mL, 1 mmol) was added to a solution of **5** (0.48 g, 1 mmol) dissolved in 6 mL of dry THF. After 10 min, DMTMM[4] (0.28 g, 1.2 mmol) was added and the reaction mixture stirred at room temperature for 12 h. The reaction was concentrated under vacuum and the residue was dissolved with ethyl acetate. The organic solution was firstly washed with 2 M NaOH and secondly with 0.5 M HCl and then concentrated in vacuo. The residue was submitted to purification by silica gel chromatography eluting with hexane/ethyl acetate and the pure product was recovered as an oil in an 85% yield. ^{1}H NMR (as a 4:1 mixture of conformers A/B) δ: 0.9 (d, 3H, $J = 6.9$); 0.93 (d, 3H, $J = 6.9$); 0.99 (d, 3H, $J = 6.6$); 1.02 (d, 3H, $J = 6.6$); 1.16–1.95 (m, 10H); 1.3 (A) (t, 3H, $J = 6.9$); 1.31 (B) (t, 3H, $J = 6.9$); 1.46 (s, 3H); 1.6 (s, 3H); 2.01 (m, 1H); 2.3 (m, 1H); 2.4 (A) (dd, 1H, $J = 1.2$, 15.3); 2.55 (A) (d, 1H, $J = 15$); 2.7 (B) (d, 1H, $J = 15$); 2.87 (B) (d, 1H, $J = 15$); 3.07 (A) (d, 1H, $J = 14.7$); 3.19 (A) (d, 1H, $J = 15.3$); 3.74 (m, 1H); 4.02–4.30 (m, 3H); 4.24 (d, 1H, $J = 13.5$); 4.45 (d, 1H, $J = 13.5$); 4.53 (dd, 1H, $J = 6.9$, 8.7); 5.05 (m, 2H); 5.92 (A) (s, 1H); 6.03 (B) (s, 1H); 6.44 (A) (d, 1H, $J = 7.8$); 6.59 (B) (d, 1H, $J = 7.8$); 6.7 (A) (d, 1H, $J = 9.3$); 7.9 (B) (d, 1H, $J = 9.3$). ^{13}C NMR δ: 14.1, 16.2, 17.6, 18.4, 19.3, 21.6, 24.7, 25.5, 28.5, 30.7, 31.2, 32.7, 32.8, 45.0, 47.3, 48.3, 52.9, 55.5, 58.6, 58.8, 61.4, 68.3, 108.5, 141.2, 157.4, 170.0, 171.2, 171.7, 173.6. $[\alpha]_D = -34.3$ (c 0.7, $CHCl_3$). Anal. Calcd for $C_{30}H_{46}N_5O_5$: C, 64.72; H, 8.33; N, 12.58. Found: C, 64.48; H, 8.34; N, 12.60.

6.1.4. 1-[(1-Carbonyl-2(*S*)-isopropyl-3,8-diaza-4,7-dioxo-6(*R*)-*N*-acetamido-6-methyl-9(*S*)-isopropyl)ethyl decanoate]-2(*S*)-methyl-4-methylene-2-cyclohexylcarbamoylpyrrolidine hydrochloride, 7. HCl 0.5 M (1 mL) was added to a solution of **6** (0.134 g, 0.24 mmol) dissolved in ethanol (2 mL) and the reaction mixture was stirred at room temperature. After 12 h ethanol was evaporated, the residue extracted with CH_2Cl_2 and the organic solution dried over $CaCl_2$. After filtration, to the organic solution was added triethylamine (0.14 mL, 0.96 mmol) and then cooled to −10 °C. Acetyl chloride (0.03 mL, 0.42 mmol) was added and after 10–15 min the cooling bath removed. The reaction mixture was stirred for 3 h and then the organic solvent was evaporated under vacuum. The residue was dissolved in ethyl acetate, the organic solution washed with 2 M HCl and then dried over Na_2SO_4. After evaporation in vacuo to dryness, the residue was submitted to silica gel chromatography eluting with hexane/ethyl acetate and the product was recovered as a wax in a 70% overall yield. ^{1}H NMR (as a 4:1 mixture of conformers A/B) δ: 0.96 (d, 3H, $J = 6.6$); 0.98 (d, 3H, $J = 6.6$); 1.0 (d, 3H, $J = 6.6$); 1.04 (d, 3H, $J = 6.6$); 1.1–2.0 (m, 10H); 1.29 (t, 3H, $J = 6.9$); 1.62 (s, 3H); 1.67 (s, 3H); 2.04 (s, 3H), 2.05 (m, 1H); 2.22 (m, 1H); 2.41 (A) (d, 1H, $J = 14.7$); 2.66 (A) (d, 1H, $J = 14.4$); 3.04 (A) (d, 1H, $J = 14.4$); 3.16 (B) (d, 1H, $J = 14.4$); 3.23 (A) (d, 1H, $J = 15.3$); 3.73 (m, 1H); 4.1–4.6 (m, 6H); 5.08 (m, 2H); 6.5 (A) (d, 1H, $J = 8.1$); 6.53 (B) (d, 1H, $J = 8.1$); 6.87 (B) (d, 1H, $J = 9$); 6.96 (A) (d, 1H, $J = 8.1$); 7.36 (B) (s, 1H); 7.39 (A) (s, 1H); 7.98 (A) (d, 1H, $J = 8.7$); 8.26 (B) (d, 1H, $J = 7.8$). ^{13}C NMR δ: 14.1, 17.7, 18.1, 19.0, 19.3, 21.4, 22.9, 24.0, 24.7, 25.5, 30.5, 30.8, 32.7, 32.8, 42.3, 44.9, 48.4, 52.9, 56.8, 57.8, 58.7, 61.0, 68.4, 108.4, 141.4, 170.6, 170.8, 171.1, 171.6, 171.8, 173.5. The product was not isolated in sufficiently pure form for elemental analysis and to measure the specific rotation.

6.1.5. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino]-3-methyl-butyryl}-4-methylene-pyrrolidine-2(*R*)-carboxylic acid ethyl-ester, 10. Compound **10** was prepared by reacting the activated ester **2** (1.27 g, 3 mmol) with **8** (0.87 g, 3 mmol) and following the procedure used for **4**. The product was recovered as an oil in an 85% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR δ: 0.89 (d, 3H, $J = 6.9$); 0.91 (d, 3H, $J = 6.9$); 0.94 (d, 3H, $J = 6.9$); 1.01 (d, 3H, $J = 6.9$); 1.29 (m, 6H); 1.48 (s, 3H); 2.0 (m, 1H); 2.29 (m, 1H); 2.54 (d, 1H, $J = 14.1$); 2.68 (m, 1H); 2.96 (m, 1H); 3.06 (m, 1H, $J = 14.1$); 4.05 (m, 1H); 4.18 (m, 2H); 4.26 (d, 1H, $J = 14.1$); 4.36 (d, 1H, $J = 14.1$); 4.62 (m, 1H); 5.09 (m, 2H); 6.01 (s, 1H); 6.72 (d, 1H, $J = 8.1$). ^{13}C NMR δ: 13.9, 14.0, 16.1, 17.6, 18.2, 19.2, 28.1, 30.7, 31.4, 35.2, 47.2, 51.1, 54.9, 58.4, 58.6, 61.0, 61.2, 108.8, 141.8, 157.2, 169.7, 170.6, 171.0, 173.3. The product was not isolated in a sufficiently pure form for elemental analysis and to measure the specific rotation.

6.1.6. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino]-3-methyl-butyryl}-4-methylene-pyrrolidine-2(*R*)-carboxylic acid, 11. Compound **11** was obtained from intermediate **10** and following the procedure described for **5**. The product was recovered as an oil in an 80% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR (CD_3OD) δ: 0.93 (d, 3H, $J = 6.9$); 0.94 (d, 3H, $J = 6.9$); 0.96 (d, 3H, $J = 6.9$); 1.04 (d, 3H, $J = 6.9$); 1.29 (t, 3H, $J = 6.9$); 1.43 (s, 3H); 1.54 (s, 3H); 2.03 (m, 1H); 2.5 (m, 1H); 2.63 (d, 2H, $J = 15$); 2.92 (d, 1H, $J = 15$); 2.98 (d, 1H, $J = 15$); 4.02 (d, 1H, $J = 3.3$); 4.05–4.25 (m, 2H); 4.35 (dd, 1H, $J = 1.5$, 13.8); 4.44 (d, 1H, $J = 8.4$); 4.52 (d, 1H, $J = 13.8$); 5.25 (m, 2H). ^{13}C NMR (CD_3OD) δ: 14.9, 17.5, 18.9, 19.0, 19.7, 21.8, 29.4, 32.3, 32.5, 46.0, 53.7, 57.2, 59.8, 59.9, 62.5, 67.5, 109.4, 143.6, 159.4, 171.5, 172.4, 176.1, 176.3. $[\alpha]_D = -16.9$ (c 0.9, $CHCl_3$). Anal. Calcd for $C_{23}H_{36}N_4O_6$: C, 59.46; H, 7.81; N, 12.06. Found: C, 59.23; H, 7.84; N, 12.10.

6.1.7. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino]-3-methyl-butyryl}-2(*R*)-methyl-4-methylene-pyrrolidine-2-carboxylic acid ethylester, 12. Compound **12** was prepared by reacting the activated ester **2** with **9** and following the procedure used for **10**. The product was recovered as an oil in an 80% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR δ: 0.88 (d, 3H, $J = 7$); 0.89 (d, 3H, $J = 6.6$); 0.93 (d, 3H, $J = 6.6$); 1.0 (d, 3H, $J = 7$);

1.24 (t, 3H, $J = 7$); 1.28 (t, 3H, $J = 7$); 1.48 (s, 3H); 1.53 (s, 3H); 1.95 (m, 1H); 2.3 (m, 1H); 2.5 (d, 1H, $J = 15.2$); 2.53 (d, 1H, $J = 14.4$); 2.88 (d, 1H, $J = 15.2$); 3.05 (d, 1H, $J = 14.4$); 3.98–4.30 (m, 6H); 4.5 (m, 2H); 5.06 (m, 2H); 5.94 (s, 1H); 6.63 (d, 1H, $J = 9.2$). ^{13}C NMR δ: 14.1, 16.1, 17.7, 18.3, 19.1, 21.1, 28.4, 30.7, 31.7, 44.6, 47.5, 52.2, 55.1, 58.6, 58.8, 61.1, 61.4, 65.9, 108.8, 141.6, 169.7, 169.9, 172.7, 173.4. The product was not isolated in a sufficiently pure form for elemental analysis and to measure the specific rotation.

6.1.8. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino]-3-methyl-butyryl}-2(*R*)-methyl-4-methylene-pyrrolidine-2-carboxylic acid, 13. Compound **13** was obtained from intermediate **12** and following the procedure described for **11**. The pure product was recovered as an oil in an 80% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR δ: 0.86 (d, 3H, $J = 6.8$); 0.92 (d, 6H, $J = 6.8$); 1.03 (d, 3H, $J = 6.8$); 1.26 (t, 3H, $J = 7.2$); 1.45 (s, 3H); 1.54 (s, 3H); 2.02 (m, 1H); 2.25 (m, 1H); 2.47 (d, 1H, $J = 15.8$); 2.6 (d, 1H, $J = 15.8$); 2.96 (d, 1H, $J = 15.8$); 3.04 (s, 1H, $J = 15.8$); 3.96–4.35 (m, 4H); 4.45 (m, 2H); 5.02 (m, 2H); 7.19 (s, 1H); 7.5 (d, 1H, $J = 9.2$). ^{13}C NMR δ: 13.9, 15.9, 17.8, 18.1, 18.9, 20.8, 20.9, 28.6, 30.3, 30.9, 44.4, 46.5, 52.3, 55.6, 58.0, 58.4, 61.1, 66.0, 108.4, 141.7, 157.8, 170.1, 170.5, 174.9. $[\alpha]_D = -48.6$ (*c* 0.7, $CHCl_3$). Anal. Calcd for $C_{24}H_{38}N_4O_6$: C, 60.23; H, 8.0; N, 11.71. Found: C, 60.44; H, 7.98; N, 11.69.

6.1.9. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino}-3-methyl-butyryl}-4-methylene-pyrrolidine-2(*R*)-carboxylic acid cyclohexyl amide, 14. Compound **14** was obtained by treating intermediate **11** (0.93 g, 2 mmol) with cyclohexylamine (0.25 mL, 2.2. mmol) in the presence of DMTMM[4] (0.72 g, 2.6 mmol) and following the procedure described for **6**. The pure product was recovered as an oil in an 83% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR δ: 0.90 (d, 3H, $J = 6.8$); 0.97 (d, 3H, $J = 6.8$); 0.99 (d, 3H, $J = 6.8$); 1.01 (d, 3H, $J = 6.8$); 1.1–1.9 (m, 10H); 1.33 (t, 3H, $J = 7.2$); 1.5 (s, 3H); 2.02 (m, 1H); 2.25 (m, 1H); 2.69 (d, 1H, $J = 14.6$); 2.75 (m, 1H); 2.86 (d, 1H, $J = 14.8$); 2.95 (d, 1H, $J = 15$); 3.67 (m, 1H); 4.01 (m, 1H); 4.05–4.35 (m, 4H); 4.42 (d, 1H, $J = 14.2$); 4.7 (d, 1H, $J = 8.8$); 5.08 (m, 2H); 6.14 (s, 1H); 6.67 (d, 1H, $J = 8.6$); 7.38 (d, 1H, $J = 7.2$). ^{13}C NMR δ: 14.1, 16.5, 18.4, 18.6, 19.2, 24.2, 24.9, 25.4, 27.9, 29.7, 30.5, 30.9, 31.2, 32.5, 35.6, 47.0, 48.7, 51.4, 56.9, 58.8, 60.0, 61.6, 109.0, 142.1, 157.7, 169.7, 171.3, 173.2. $[\alpha]_D = +41.0$ (*c* 0.4, $CHCl_3$). Anal. Calcd for $C_{29}H_{44}N_5O_5$: C, 64.18; H, 8.17; N, 12.9. Found: C, 63.95; H, 8.19; N, 12.95.

6.1.10. 1-{2(*S*)-[2-(6-Ethoxy-5(*S*)-isopropyl-2(*R*)-methyl-3-oxo-2,3,4,5-tetrahydro-pyrazin-2-yl)-acetylamino}-3-methyl-butyryl}-2(*R*)-methyl-4-methylene-pyrrolidine-2-carboxylic acid cyclohexyl amide, 15. Compound **15** was obtained from intermediate **13** and following the procedure described for **14**. The product was recovered as an oil in an 85% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR δ: 0.94 (d, 3H, $J = 6.8$); 1.02 (m, 9H); 1.3 (t, 3H, $J = 7$); 1.5 (s, 3H); 1.7 (s, 3H); 1.1–1.9 (m, 10H); 2.0 (m, 1H); 2.15 (m, 1H); 2.52 (d, 1H, $J = 15$); 2.69 (d, 1H, $J = 14.6$); 2.86 (d, 1H, $J = 14.6$); 3.02 (d, 1H, $J = 15$); 3.65 (m, 1H); 4.0–4.4 (m, 5H); 4.55 (d, 1H, $J = 13.5$); 5.02 (m, 2H); 6.02 (s, 1H); 6.44 (d, 1H, $J = 8$). ^{13}C NMR δ: 14.0, 16.4, 18.4, 18.8, 18.9, 21.9, 24.9, 25.0, 25.5, 27.7, 30.8, 31.2, 32.6, 46.2, 46.7, 48.6, 53.2, 57.3, 58.6, 61.5, 68.4, 108.4, 141.1, 157.7, 169.0, 171.3, 171.8, 173.1. The product was not isolated in a sufficiently pure form for elemental analysis and to measure the specific rotation.

6.1.11. 1-[(1-Carbonyl-2(*S*)-isopropyl-3,8-diaza-4,7-dioxo-6(*R*)-*N*-acetamido-6-methyl-9(*S*)-isopropyl)ethyl decanoate]-4-methylene-2(*R*)-cyclohexylcarbamoylpyrrolidine hydrochloride, 16. Compound **16** was obtained from intermediate **14** and following the procedure described for **7**. The pure product was recovered as a wax in an 85% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR δ: 0.9–1.05 (m, 12H); 1.29 (t, 3H, $J = 7.2$); 1.71 (s, 3H); 2.07 (s, 3H); 1.05–2.0 (m, 10H); 2.08 (m, 1H); 2.27 (m, 1H); 2.63 (d, 1H, $J = 14.2$); 2.83 (m, 1H); 2.96 (d, 1H, $J = 14.2$); 3.13 (d, 1H, $J = 14.2$); 3.75 (m, 1H); 4.18–4.38 (m, 4H); 4.43 (m, 2H); 4.73 (m, 1H); 5.09 (m, 2H); 6.75 (d, 1H, $J = 8.4$); 7.07 (s, 1H); 7.13 (d, 1H, $J = 6.3$); 7.88 (d, 1H, $J = 8$, 7). ^{13}C NMR δ: 14.1, 17.7, 18.4, 19.0, 19.3, 23.6, 23.8, 24.9, 25.4, 29.9, 30.9, 32.6, 32.8, 35.8, 42.9, 48.5, 51.3, 57.4, 57.7, 59.2, 60.0, 61.1, 108.8, 142.0,1, 169.6, 171.0, 171.1, 171.7, 171.8, 173.7. $[\alpha]_D = +75.7$ (*c* 0.5 $CHCl_3$). Anal. Calcd for $C_{31}H_{47}N_5O_7$: C, 61.88; H, 7.87; N, 11.64. Found: C, 62.15; H, 7.85; N, 11.62.

6.1.12. 1-[(1-Carbonyl-2(*S*)-isopropyl-3,8-diaza-4,7-dioxo-6(*R*)-*N*-acetamido-6-methyl-9(*S*)-isopropyl)ethyl decanoate]-2(*R*)-methyl-4-methylene 2-cyclohexylcarbamoylpyrrolidine hydrochloride, 17. Compound **17** was obtained from intermediate **15** and following the procedure described for **7**. The product was recovered as a wax in an 83% yield after elution by silica gel chromatography with hexane/ethyl acetate. ^{1}H NMR δ: 0.92 (d, 3H, $J = 6.9$); 0.96 (d, 3H, $J = 6.9$); 0.98 (d, 6H, $J = 6.6$); 1.27 (t, 3H, $J = 7.2$); 1.64 (s, 3H); 1.68 (s, 3H); 1.1–1.9 (m, 10H); 2.02 (m, 1H); 2.23 (m, 1H); 2.53 (d, 1H, $J = 15.6$); 2.64 (d, 1H, $J = 14.4$); 2.97 (m, 2H); 3.7 (m, 1H); 4.16 (m, 3H); 4.27 (d, 1H, $J = 13.8$); 4.4 (m, 1H); 4.57 (d, 1H, $J = 13.8$); 5.04 (m, 2H); 6.54 (d, 1H, $J = 8.1$); 7.24 (d, 1H, $J = 6.6$); 7.46 (s, 1H); 7.86 (d, 1H, $J = 8.1$). ^{13}C NMR δ: 14.1, 17.6, 18.5, 18.9, 19.0, 21.7, 23.9, 24.9, 25.5, 29.9, 30.8, 32.6, 32.7, 43.4, 46.2, 48.6, 53.1, 57.6, 57.8, 59.5, 61.1, 65.8, 68.4, 108.6, 140.9, 170.7, 171.2, 171.6, 171.7, 172.0, 173.9. The product was not isolated in a sufficiently pure form for elemental analysis and to measure the specific rotation.

6.2. Computational details

The computational strategy used here is a high-temperature quenched molecular dynamics (QMD) approach. The QMD protocol, which has been used over the last decade to produce a reasonable molecular structure of a variety of peptides, allows us to search the conformational

space of the pseudopeptides and to locate the lowest energy minimum.

The 3D structures of the molecules were built with CORINA.[9] All the calculations were performed at the molecular mechanics level using the AMBER 8.0 package.[10] Simulations were carried out using the Gaff force field.[11] Charges were assigned to atoms using the AM1-BCC method[12] as implemented in the Antechamber package.[13] Solvation effects were incorporated using the Generalized Born Model.[14] Thus dynamics were performed with a dielectric constant $\varepsilon = 4.9$ to simulate the electrostatic effects of chloroform (the solvent where ^{1}H NMR data have been recorded).

To locate the lowest energy structure, without being trapped in local minima, we first employed a preliminary QMD simulation where the molecules were heated from 0 to 600 K in 100 ps and then, a trajectory of 5 ns was carried out at constant temperature (600 K) and constant pressure (1 atm) with an integration step of 2 fs. The SHAKE algorithm[15] was used to constrain the stretching of bonds involving hydrogen atoms. The coordinates of the pseudopeptides were saved on a trajectory file every 1 ps, giving a total of 5000 conformations for further analysis. Each of the structures obtained was energy minimized till the root mean square of the Cartesian elements of the gradient was less than 0.001 kcal mol^{-1} using a full conjugate gradient minimization and GB/SA model.[14,16]

This preliminary simulation provided the best starting structure for a new molecular dynamics at 300 K to identify intramolecular hydrogen bonds and examine the conformational space of the pseudopeptides at an ambient temperature. These dynamics were carried out for 1 ns using a time step of 0.001 ps and writing the coordinates every 0.5 ps on a trajectory file. We analyzed this file with the 'ptraj' package (an AMBER module)[10] to obtain an estimate of the lifetime of every H-bond during the simulation. The lifetime is expressed as a percentage of the existence of the H-bond during the whole simulation (distance between acceptor and donor shorter than 4 Å, and a bond angle larger than 109°). For structure **6** we have compared the results obtained with the 1 ns trajectory to those obtained with a 10 ns trajectory. Since the difference in the lifetimes was not larger than 3%, we have used trajectories of 1 ns for all the six pseudopeptides examined here.

To visualize the most important conformations of the peptides, we have carried out a cluster analysis (the MMTSB toolset was used).[17] We have clustered the conformations obtained from the dynamics by a structural similarity (using kclust and a fixed radius clustering of 1.5 Å on cartesian coordinate RMSD of heavy atoms). Clusters have been grouped together, if possible, on the basis of the similarity of the hydrogen bond pattern. For each set of clusters the most populated structures have been selected as the representative conformations of each pseudopeptide.

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