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Solid-phase synthesis of hydrazinopeptides in Boc and Fmoc strategies monitored by HR-MAS NMR

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Abstract—With the aim of finding good conditions to incorporate hydrazino moieties into peptidic sequences of biological interest, we developed two strategies towards solid-phase synthesis of hydrazinopeptides: a stepwise methodology in which a hydrazinoacid was introduced as a normal aminoacid and a semi-convergent one in which the hydrazino moiety is incorporated as a pseudodipeptide building block. HR-MAS NMR analyses were run in order to monitor the reaction and to improve the reaction conditions.
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1. Introduction

The replacement of an amide bond with that of a hydrazide bond in a peptide chain leads to the formation of novel compounds called hydrazinopeptides. It has been demonstrated that the presence of hydrazide bonds in a peptide chain can induce a specific folding conformation called a hydrazinoturn.[1] Moreover, Lelais and seebach demonstrated that hydrazinomers were very resistant to protease activity.[2] Both remarks allow consideration of the use of these pseudopeptides to mimic endogenous peptides in biological processes. In fact, only a few hydrazinopeptides have been described in the literature; Niedrich et al. synthesized nine analogues of eledoisin,[3] an octapeptide involved in vasodilatation processes and Guy et al. described some human leukocyte elastase (HLE) inhibitors containing hydrazino moiety.[4] In both cases, the overall yields varied from low to moderate. More recently, Lelais et al. synthesized a hexahydrazinopeptide in solution with an overall yield of 14%.[2]

One of the main obstacles in the synthesis of hydrazinopeptides is regioselectivity. The presence of the two nitrogen atoms makes regioselective acylation of α-hydrazinoacids difficult to perform without protection. As an example, during the study of the protection of the hydrazinoglycine ethyl ester H_2N^{β}-HN^{α}-CH_2-COOEt, Viret et al.[5] and Lecoq and Marraud[6] demonstrated that the formation of mono- and bis-acylated products strongly depends on the nature of the reactants and the procedure used. Furthermore, coupling of unprotected hydrazinoacids can give rise to byproducts such as diketopiperazine analogues or oligomers.[4] Unfortunately, very few methods allow the synthesis of orthogonally protected hydrazinoacid derivatives with good yield.

In previous papers,[7] we showed that chiral orthogonally protected hydrazinoacid derivatives could be easily prepared via a Mitsunobu reaction. More recently, we demonstrated that N^{β}-Fmoc- and N^{β}-Boc-N^{α}-*Z*-hydrazinoacid derivatives, directly suitable for SPPS, could be easily prepared in six steps with good yields starting from the corresponding α-aminoacids.[7c] The coupling reaction assays performed between N^{α}-*Z*-hydrazinoesters and *N*-Fmoc-α-aminoacids demonstrated the low reactivity of α-hydrazinoester derivatives when the N^{α} is protected by a benzyloxycarbonyl group. However, among the numerous coupling methods tested, we found that the acid fluoride method allowed the formation of hydrazinodipeptides almost quantitatively. Considering that one of the goals of this study was to incorporate the hydrazino moiety into sequences of biological interest, we decided to find conditions, which allowed the synthesis of hydrazinopeptides on a solid-phase support. To date, only the H-Asp-Tyr-Glyψ[CO-NH-NH]Ile-Leu-Gln-Ile-Asn-Ser-Arg-OH (hydrazide) decapeptide, an analogue of an HEL T-epitope, has been prepared in poor yield by a solid-phase procedure, using the step-by-step Boc/TFA/HF strategy.[8] Bocψ[CO-NH-NH]Ile-OH was incorporated using DCC/HOBt, and the coupling step was repeated three times with 3 equiv each time. It is noteworthy that, due to the presence of the unprotected α-nitrogen in the growing (hydrazide) peptide, capping with Ac_2O is precluded.

We describe in this paper, the comparative study of two original protocols. The first one, a step-by-step synthesis,

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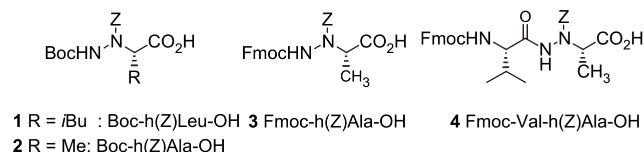


Figure 1. Boc- and Fmoc-hydrazinoacid **1**–**3** and building block **4** used in this work.

consisting of the incorporation of N^{α},N^{β}-protected hydrazinoacids **1**–**3** (Fig. 1) using a classical coupling reaction generally used for peptide synthesis. In the second one, preformed hydrazinodipeptides **4** (Fig. 1), previously described[7c] are incorporated as building blocks in a peptide chain.

2. Results and discussion

2.1. Step-by-step synthesis

To define the optimal conditions, we decided to synthesize the pseudotripeptide H-Pro-hLeu-Ala-OH (**5**) on a Boc-Ala-PAM resin using a classical Boc strategy. The formation of the amide bond Leu-Ala was performed by using the classical TBTU/HOBt/DIEA mixture. As expected, the formation of the hydrazide bond was more delicate. When using the acid fluoride method in a $NaHCO_3$/DCM mixture, we did not observe any formation of the desired product. The only product obtained was the pseudodipeptide H-h(*Z*)Leu-Ala-OH. This result clearly demonstrates that the acid fluoride coupling method found to be the best in liquid phase protocol[7c] is ineffective on solid support. Furthermore, we noticed that the low reactivity of the N^{β} renders the qualitative colorimetric tests (ninhydrin, TNBSA) useless. This remark leads us to use HR-MAS NMR (High-Resolution Magic Angle Spinning) analysis to monitor each step of the synthesis of **5**. This NMR technique allows spectral analysis of peptides that are still linked to their resin support in such a way that the completion of each coupling and deprotecting steps can be accurately and easily observed. Using this NMR technique, we were able to demonstrate that the replacement of $NaHCO_3$/DCM mixture by DIEA/DMF allowed the coupling of Fmoc-Pro-F. It is interesting to note that during all these assays, an *N*-Fmoc protected proline was used instead of a Boc one. The reasoning behind this is due to the fact that the Fmoc group is more accurately identified by NMR spectra than the Boc group. This allows more precise estimation of the yield in the last coupling reaction by calculation of the ratio between the signals of the $\gamma(CH_3)$Leu and those of the Fmoc group. After one coupling run, the incorporation of the proline aminoacid was estimated to 35% yield (Fig. 2). This result confirmed the low reactivity of the N^{β}-position and that specific conditions must be found to increase the yield of hydrazinopeptide synthesis on solid phase.

After this encouraging result, we considered the incorporation of a hydrazinoacid in a longer sequence to evaluate the impact of this insertion on the further steps of the synthesis. We decided to synthesize the PFVhAL sequence (**6**) for two main reasons. (1) This pseudopeptide, easily discernible by HR-MAS NMR analyses, does not have any functionalized lateral chain, which necessitates a protective group. (2) This sequence involved the coupling reaction between a hindered aminoacid, valine, on the unreactive N^{β} of the hydrazinoalanine moiety. First, an assay was run via the Boc strategy on a Boc-Leu-PAM resin as described in Scheme 1.

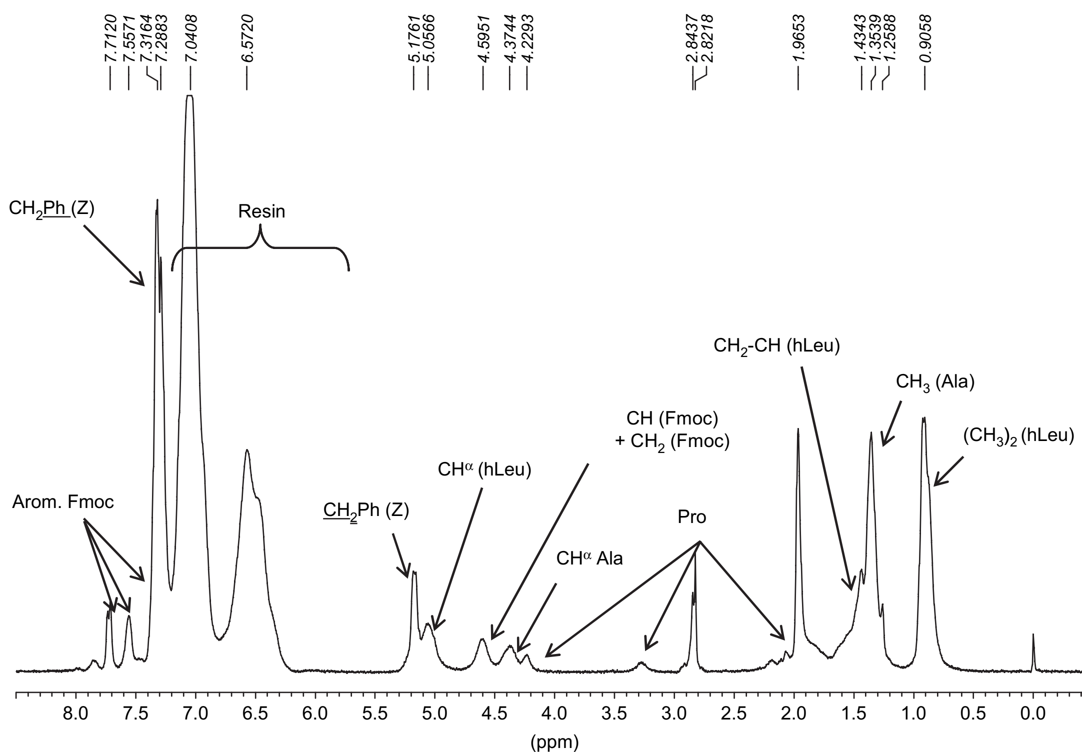
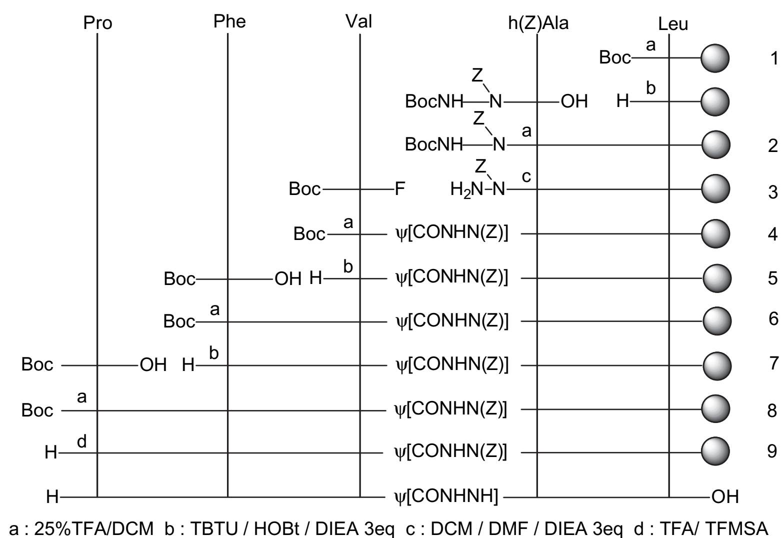


Figure 2. HR-MAS spectrum of Fmoc-Pro-hLeu-Ala-PAM resin.

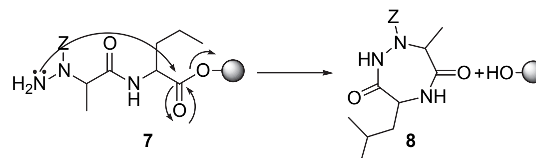


Scheme 1. Synthetic procedure for the preparation of PFVhAL in Boc strategy.

The coupling and deprotection steps were monitored by HR-MAS by visualizing the presence of the Boc signal (Fig. 3). In this assay, we replaced DMF by a 50/50 DCM/DMF mixture for the coupling of fluoride to ensure a better swelling of the resin.

The second spectrum in Figure 3 shows the simultaneous presence of the *Z* (at 5.2 ppm) and the Boc (at 1.5 ppm) groups, which confirms the linkage of the modified amino-acid Boc-h(*Z*)Ala-OH (**2**). Every coupling (spectra 2, 4, 6, 8) and deprotection (spectra 3, 5, 7, 9) steps can be qualitatively verified, respectively, by checking the presence or the absence of the Boc signal.

During these assays, some important information arose. (i) To reach a reasonable incorporation level of Boc-Val-F into the sequence, four successive couplings were required. (ii) Surprisingly, after the formation of the hydrazide bond, the further deprotection and coupling steps were problematic. (iii) After cleavage of the pseudopeptide from the resin and HPLC purification, we observed as side products, some deleted peptides particularly the H-Pro-Phe-Val-OH tripeptide. The absence of the hAla-Leu sequence could be explained by a cyclization step occurring after the removal of the Boc group from the hAla residue resulting in the formation of the perhydrotriazepine **8**. Such cyclization can be compared to those leading to the well known diketopiperazine[9] (Scheme 2). Unfortunately, we were not able to isolate this compound.



Scheme 2. Formation of perhydrotriazepane **8**.

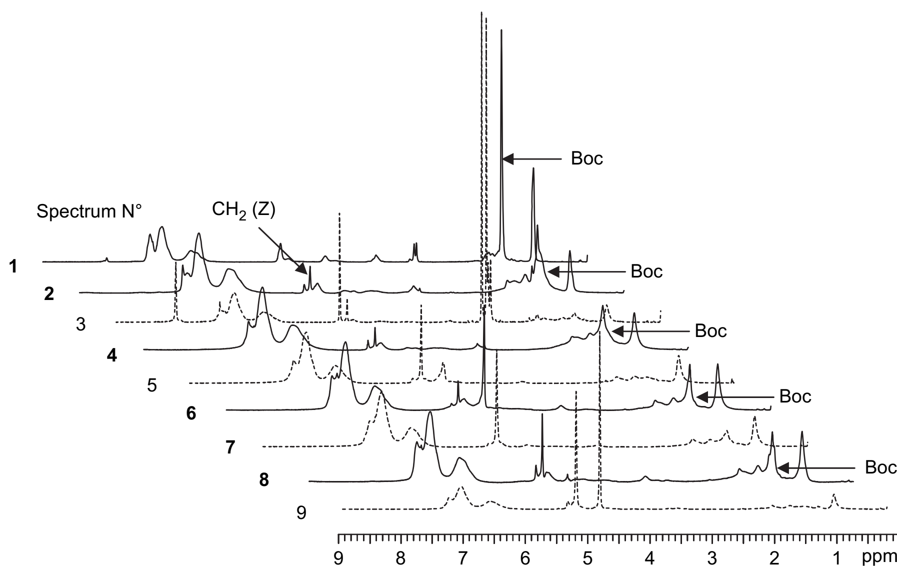
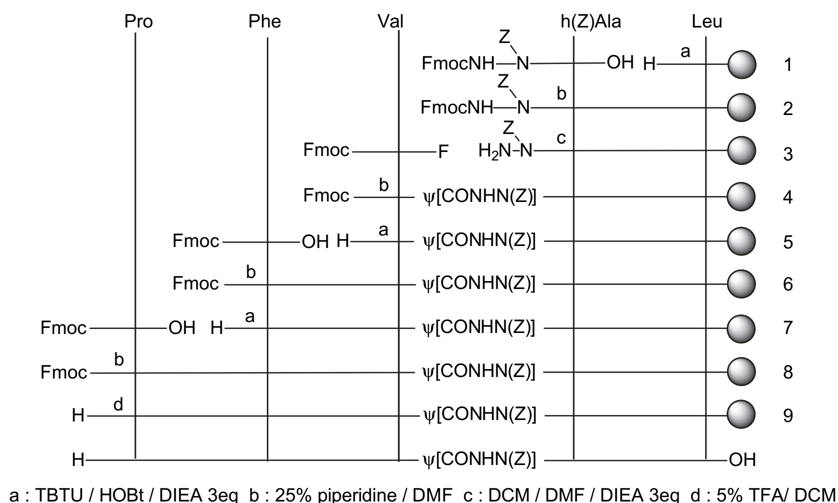


Figure 3. HR-MAS monitoring during synthesis of PFVhAL on a PAM resin.



Scheme 3. Synthetic procedure for the preparation of PFVhAL in Fmoc strategy.

To circumvent the postulated formation of perhydrotriazepine, we decided to perform the synthesis on a more hindered 2-chlorotrityl resin as recommended for preventing the formation of diketopiperazine. This kind of resin necessitated the use of the Fmoc strategy described in Scheme 3. Due to the mild conditions used for the cleavage of the peptide from the resin, the protected PFVh(*Z*)AL pseudopeptide (**9**) is expected as the final product.

This synthesis was monitored by HR-MAS and the spectra are gathered in Figure 4. The simultaneous presence of the *Z* (at 5.2 ppm) and of the Fmoc (at 7.6 and 7.8 ppm) signals on the spectrum 2 (Fig. 4) confirms the linkage of the hydrazinoacid Fmoc-h(*Z*)Ala-OH (**3**) on the *H*-Leu-2-chlorotrityl resin. The efficiency of every coupling and deprotection reactions can be monitored by HR-MAS by visualizing the presence or the absence of the signal of the Fmoc group (Fig. 4).

HR-MAS analysis demonstrated that the incorporation of the Fmoc-Val-F was delicate to perform and necessitated three successive coupling reactions in order to observe an NMR signal corresponding to the Fmoc protecting group. The PFVh(*Z*)AL was obtained after purification with a moderate yield of 13%. Keeping in mind that hydrazide bond can lead to a specific structuration called hydrazinoturn,[1] we suspected the structuration of the growing pseudopeptide to be responsible for the poor yield by decreasing the accessibility of the reactants. So, we decided to use magic mixtures[10] as solvent, which were known to have a destructurant effect on peptides. The above synthesis procedure was repeated by using a mixture of DMF/DCM/NMP (33/33/33, v/v/v) for the coupling steps and piperidine/DMF/NMP/toluene (25/25/25/25, v/v/v/v) for the deprotection of the Fmoc group. With the help of these solvent mixtures, the overall yield of pure pseudopentapeptide rose to 27%. Surprisingly, the addition of 1% Triton X-100[10] results in a dramatic decrease in the yield. Aiming to study the influence of the steric hindrance of the different amino or hydrazinoacids, we underwent the synthesis of PFh(*Z*)AVL (**10**) in place of PFVh(*Z*)AL (**9**) and we obtained the pure pseudopeptide in 36% yield. This result confirms that the coupling reaction between the hydrazine moiety and an aminoacid is strongly dependent on the steric hindrance of the side chain of the

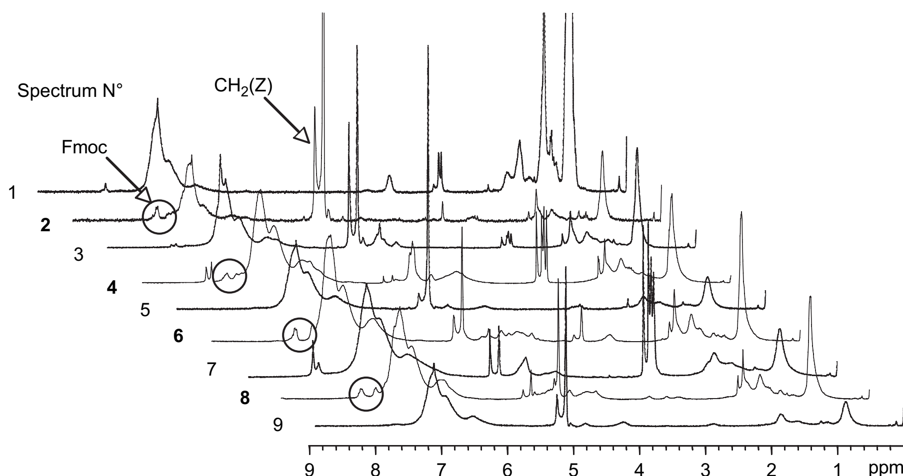
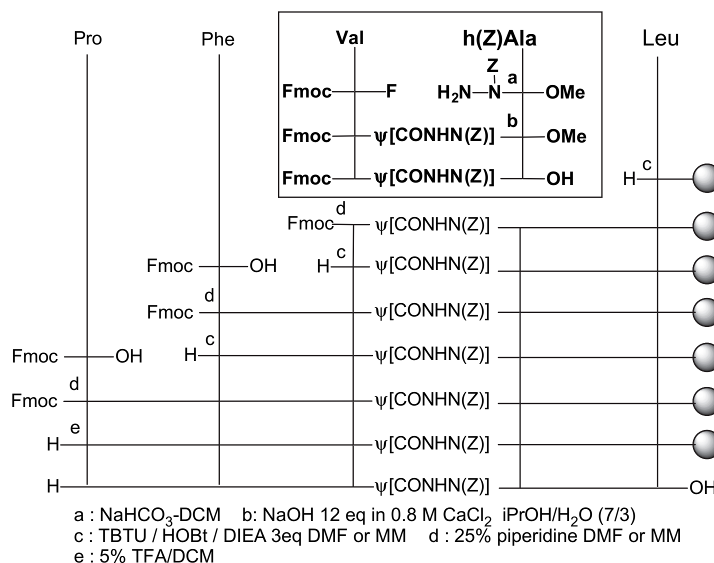


Figure 4. HR-MAS monitoring during synthesis of PFVhAL on a 2-chlorotrityl resin.



Scheme 4. Synthetic procedure for the preparation of PFVhAL (**9**) by semi-convergent methodology.

aminoacid. Considering that the deprotection of the Fmoc group in our case is a limiting step, this step-by-step strategy comprises 10 steps, with an average yield of 88% per step for PFVh(*Z*)AL (**9**) and 90% per step for PFh(*Z*)AVL (**10**).

2.2. Semi-convergent synthesis

Hydrazinopeptides could be obtained using another strategy based on the incorporation of a preformed synthon of general formula Fmoc-Xaa-h(*Z*)Xbb-OH into the growing pseudopeptide. As we previously described, these building blocks can be easily synthesized in liquid phase.[7c] To test the efficiency of this strategy, the synthesis of **9** was run on a Leu-Wang resin as depicted in Scheme 4.

The building block Fmoc-Val-(*Z*)Ala-OH (**4**) can be anchored by using a classical coupling method. When DMF was used as solvent, an overall 32% yield was obtained while the use of magic mixtures led to 46% (regardless of the resin substitution) of pure pseudopentapeptide (**9**). This overall yield corresponds to an average yield of 87% per step.

3. Conclusion

We demonstrated that the incorporation of hydrazino bond in a growing peptide chain can be performed on solid phase by a step-by-step strategy or by direct incorporation of a hydrazinodipeptides. With the help of HR-MAS NMR analyses, we were able to follow the progression of the synthesis and demonstrated that the use of magic solvents improves the overall yield of the synthesis.

4. Experimental

4.1. General

Tetrahydrofuran was dried by distillation over sodium benzophenone ketyl. Unless otherwise stated, reagents were purchased from chemical companies and used without prior purification. Reactions were monitored by thin-layer chromatography (TLC) using aluminium-backed silica gel plates (Macherey–Nagel ALUGRAM® SIL G/UV_{254}). TLC spots were viewed under ultraviolet light and by heating the plate after treatment with a staining solution of phosphomolybdic acid. Product purifications were performed using Geduran 60 H Silica Gel (63–200 mesh). Reagent grade solvents were used as received. The Boc-Ala-Merrifield resin, Boc-Leu-PAM resin, *H*-Leu-chlorotrityl resin, Boc-aminoacids, Fmoc-aminoacids, 1-hydroxybenzotriazole (HOBt), *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TBTU) and diisopropylethylamine (DIEA) were purchased from Senn Chemicals or Novabiochem. Boc-h(*Z*)-Ala-OH and Fmoc-h(*Z*)Ala-OH were prepared as previously described in our laboratory.[7c] Solid-phase hydrazinopeptide syntheses were performed on a multichannel peptide synthesizer PSP 4000[11] according to a classical Fmoc/^{t}Bu and Boc methodology. The peptides were purified by reverse phase high-performance liquid chromatography (HPLC) using a preparative HPLC system (Waters Corp., Milford, MA, USA) on an Interchrom UP5 ODB.25 M Uptisphere 5 μm column (250×10 mm). Peptides were eluted with a gradient of solution A (water containing 0.1% of TFA) and solution B (20% of water in acetonitrile with 0.1% of TFA). The purity of the peptide was checked by analytical HPLC, which was run on a Merck apparatus (Darmstadt, Germany) using an Interchrom UP10 ODB.25 K Uptisphere 5 μm column (250×4.6 mm). ^{1}H and ^{13}C NMR (300 MHz) spectra were recorded on a Bruker Advancer 300. Multiplicities are reported as follow: s=singlet, t=triplet, q=quadruplet, m=multiplet. Electron Impact Mass Spectra were performed on a ProMALDI/FTMS apparatus in the 'Laboratoire de Spectrométrie de Masse et de Chimie Laser—Université de Metz—France'. Electron spray ionization mass spectra (ESI-MS) were recorded on an SCIEX API 150EX single quadrupole spectrometer (Sciex, Toronto, Canada).

4.2. HR-MAS NMR experiments

Resin was washed twice with CH_2Cl_2 and filtered. Then 5 mg of the resin was loaded in 4 mm rotor and swollen with

$CDCl_3$ in which tetramethylsilane (TMS) was used as internal reference. All NMR experiments were performed at 298 K on a 300 MHz spectrometer equipped with a 4 mm HR-MAS probe using a 2500 Hz spinning rate. ^{1}H NMR spectra were recorded with Carr–Purcell–Meiboom–Gill experiments (CPMG)[12] with 64 scans.

4.3. Synthesis of the pseudotripeptide PhLA (5)

The synthesis was performed using Boc-Ala-Merrifield resin (0,7 mmol/g). Boc-h(*Z*)Leu-OH (**1**) (3 equiv) was activated using HBTU/HOBT/DIEA (3 equiv, 3 equiv, 9 equiv) in DMF. After the deprotection of the Boc group using 25% TFA in DCM, Boc-Pro-F (3 equiv) was added with DIEA (9 equiv) in DMF. HR-MAS spectrum was recorded. After the N-terminal deprotection, the peptide resin was washed twice with dichloromethane and dried under vacuum. A standard cleavage with a mixture of 5% of TFMSA in trifluoroacetic acid (TFA) for 2 h afforded the crude peptide, which was lyophilized and purified by HPLC with a gradient from 0 to 50% of solution B for 40 min at a flow rate of 2 ml/min with UV detection at 254 nm. After removal of the solvents, the purified compound was lyophilized and analyzed by mass spectrometry and NMR. A single peptide peak was detected after HPLC and mass spectrometry revealed a peptide mass of 314.88 (for $C_{14}H_{27}N_4O_4$ $[M+H]^+$), virtually identical to the calculated mass of 315.20. ^{1}H NMR (1D, COSY and TOCSY) was in agreement with the sequence of the peptide, see Table 1.

Table 1. ^{1}H NMR (DMSO-d_6): δ(ppm) PhLA (**5**)

	$N^{\beta}H$	$N^{\alpha}H$	α	β	γ	δ
Pro			4.33	2.17	1.77	3.16
h(Z)Leu	8.67	8.03	4.75	1.63	1.67	0.90, 0.87
Ala		7.91	4.51	1.42		

4.4. Synthesis of the pseudopentapeptide PFVhAL (6) in Boc strategy

The synthesis was performed using Boc-Leu-PAM resin (0.7 mmol/g). The aminoacids were activated using HBTU/HOBT/DIEA (3 equiv, 3 equiv, 9 equiv) in DMF except for the acid fluoride, which was added with DIEA (9 equiv) in DMF/DCM. Each coupling was tripled to prevent deletion. Typically, the coupling reactions were complete within 6 h (three coupling reaction of 2 h). The deprotection of the Boc group was achieved with 25% TFA in DCM and each deprotection was tripled. After N-terminal deprotection, the pseudopeptide resin was washed twice with dichloromethane and dried under vacuum. A standard cleavage with a mixture of 5% of TFMSA in trifluoroacetic acid (TFA) for 2 h afforded the crude peptide, which was lyophilized and purified by HPLC with a gradient from 5 to 40% of solution B for 25 min at a flow rate of 4 ml/min with UV detection at 230 nm. After removal of the solvents, the purified compound was lyophilized and analyzed by mass spectrometry and NMR. A single peptide peak was detected after HPLC and mass spectrometry revealed a peptide mass of 560.16 (for $C_{28}H_{45}N_6O_6$ $[M+H]^+$), virtually identical to the calculated mass of 560.33. ^{1}H NMR (1D, COSY and TOCSY) was in agreement with the sequence of the peptide, see Table 2.

Table 2. ^{1}H NMR (DMSO-d_6): δ(ppm) PFVhAL (**6**)

	$N^{\beta}H$	$N^{\alpha}H$	α	β	γ	δ
Pro			3.97	2.19	1.72	3.61
Phe		8.61	4.69	3.05, 2.80		
Val		8.18	4.02	1.82	0.81	
hAla	9.47	5.16	3.35	1.11		
Leu		8.15	4.21	1.65	1.56	0.85

4.5. Synthesis of pseudopentapeptides PFVh(Z)AL (9) and PFh(Z)AVL (10) in Fmoc strategy

The synthesis was performed using *H*-Leu-2-chlorotrityl resin (0.99 mmol/g). The aminoacids were activated using HBTU/HOBT/DIEA (3 equiv, 3 equiv, 3 equiv) in DMF or in magic mixture DMF/DCM/NMP (33/33/33, v/v/v), except for the acid fluoride, which was added with DIEA (9 equiv) in DMF/DCM or in magic mixture. Each coupling was tripled to prevent deletion. For the semi-convergent synthesis, the coupling of the building block was achieved as for a usual aminoacid. Typically, the coupling reactions were complete within 6 h (three coupling reaction of 2 h). The deprotection of the Fmoc group was achieved with 25% piperidine in DMF or in magic mixture piperidine/DMF/NMP/toluene (25/25/25/25, v/v/v/v). Each deprotection was quadrupled. After N-terminal deprotection, the pseudopeptide resin was washed twice with dichloromethane and dried under vacuum. A standard cleavage with a mixture of 5% TFA in DCM for 2 h afforded the crude peptide, which was lyophilized and purified by HPLC with a gradient from 5 to 100% of solution for 35 min at a flow rate of 4 ml/min with UV detection at 230 nm. After removal of the solvents, the purified compound was lyophilized and analyzed by mass spectrometry and NMR.

For PFVh(*Z*)AL (**9**), mass spectrometry revealed a peptide mass of 695.60 (for $C_{36}H_{51}N_6O_8$ $[M+H]^+$), virtually identical to the calculated mass of 695.37. ^{1}H NMR (1D, COSY and TOCSY) was in agreement with the sequence of the peptide, see Table 3.

For PFh(*Z*)AVL (**10**), mass spectrometry revealed a peptide mass of 695.40 (for $C_{36}H_{51}N_6O_8$ $[M+H]^+$), virtually identical to the calculated mass of 695.37. ^{1}H NMR (1D, COSY

Table 3. ^{1}H NMR (DMSO-d_6): δ(ppm) PFVh(Z)AL (**9**)

	$N^{\beta}H$	$N^{\alpha}H$	α	β	γ	δ
Pro			4.00	2.17	1.77	3.06
Phe		8.65	4.67	3.04		
Val		8.30	4.27	1.97	0.94, 0.80	
h(Z)Ala	8.67	CH_2(Z) 5.06, Ph(Z) 7.33	4.29, 4.46, 4.70	1.23		
Leu		8.30	4.17	1.57	1.50, 1.49	0.84, 0.76

Table 4. ^{1}H NMR (DMSO-d_6): δ(ppm) PFh(*Z*)AVL (**10**)

	$N^{\beta}H$	$N^{\alpha}H$	α	β	γ	δ
Pro			4.00	2.17	1.77	3.06
Phe			*4.67*	*3.04*		
h(Z)Ala		CH_2(Z) 5.06, Ph(Z) 7.33	4.29, 4.46, 4.70	1.23		
Val		8.10	4.10	1.97	0.94, 0.80	

and TOCSY) was in agreement with the sequence of the peptide, see Table 4.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2007.07.038.

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