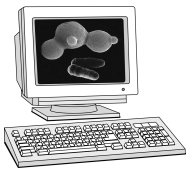
Research Article

Journal of PeptideScience

Received: 17 July 2009 Revised: 8 October 2009 Accepted: 18 October 2009 Published online in Wiley Interscience: 15 December 2009

(www.interscience.com) DOI 10.1002/psc.1203

# Slightly modifying pseudoproline dipeptides incorporation strategy enables solid phase synthesis of a 54 AA fragment of caveolin-1 encompassing the intramembrane domain



**Yves-Marie Coïc,[a]* Charlotte Le Lan,[b] Jean-Michel Neumann,[b‡] Nadège Jamin[b] and Françoise Baleux[a]**

**This work contributes to highlight the benefits of pseudoproline dipeptides introduction in difficult SPPS. We show how a slight modification in the positioning choice conditioned the synthesis achievement of a 54 amino acid long caveolin-1 peptide encompassing the intramembrane domain. Furthermore, we report a side reaction correlated with the coupling steps and generating truncated fragments with a mass deviation of +42 Da. Considering the need of structural data for membrane proteins, most of which are considered as prevalent therapeutic targets, chemical synthesis provides an interesting alternative pathway to obtain hydrophobic domains by pushing back the frontiers of conventional RP methods of purification.** 

*Supporting information may be found in the online version of this article*

**Keywords:** pseudoproline dipeptide; intramembrane peptide; RP-HPLC; SPPS; HATU; truncated peptide; side reaction; mass deviation

## Introduction

By their involvement in many biological processes, membrane proteins represent most of drug targets [1,2], despite their minor representation (around 30%) in the human genome with regards to the cytosoluble proteins [3]. The determination of their conformations and dynamics upon interaction with the plasma membrane and natural ligands could lead to the concept and design of activator or inhibitor drugs. Unfortunately, the determination of 3D structures by spectroscopic methods such as NMR or X-ray diffraction requires large concentrations of purified proteins. Because traditional expression methodologies often lead to the precipitation of most membrane protein and fail to yield a sufficient amount of material, chemical synthesis provide an alternative pathway to obtain membrane-spanning segments [4,5] or, scarcely, whole short transmembrane proteins [6]. One of the main advantages of chemical strategy is to obtain an homogeneous batch of material, devoid of extramotif such as affinity tag (e.g. poly(His)-tag) appended to expressed proteins. Moreover, according to the scientific purpose, specific introduction of labelled or modified amino acids in the native sequence can be chemically achieved. The main difficulty in applying conventional technical procedures of purification results from the hydrophobic nature of the intramembrane domains. Most soluble synthetic peptides are usually solubilised with aqueous buffers and purified by RP-HPLC using a gradient of acetonitrile. On the contrary, membrane peptides show poor water solubility and furthermore, the elution from the RP columns requires a high proportion of organic solvents [5–9]. In those conditions, it appears problematic to isolate the target peptide from coeluting truncated fragments. Success in obtaining purified hydrophobic peptides is thus strictly dependant of the crude peptide quality, which means a good yield at each coupling step and the correlative minimization of truncated fragments. One of the most effective improvements realised over the last years in difficult peptide synthesis was the insertion of pseudoproline (oxazolidine) dipeptides during the elongation of Ser- or Thr-containing peptides [10]. The incorporation of pseudoproline dipeptides leads to a better solvation of the growing peptidic chain by disrupting the formation of aggregative secondary structures. Those building blocks provide a successful tool to achieve the synthesis of difficult sequences for which conventional strategy failed [11–14]. Moreover, as shown by comparative synthetic yields, the use of pseudoproline analogues was found to be superior to 2-hydroxy-4-methoxybenzyl backbone protection [15]. Here we report how a fine modification in the incorporation strategy of pseudoproline dipeptides allowed the purification of a hydrophobic peptide using borderline RP chromatography

* *Correspondence to: Yves-Marie Coïc, Unité de Chimie des Biomolécules, URA CNRS 2128, Institut Pasteur, 28 rue du Docteur Roux, F-75724, Paris Cedex 15, France. E-mail: yves-marie.coic@pasteur.fr*

a *Institut Pasteur, Unité de Chimie des Biomolécules, URA CNRS 2128, F-75724, Paris, France*

b *CEA, iBiTecS, URA CNRS 2096, F-91191, Gif sur Yvette, France*

‡ *Deceased.*

Journal of PeptideScience

methods, by lowering the generation of predominant truncated chains during synthesis. Furthermore, we report a side reaction yielding a +42 adduct selectively observed on the truncated peptides and correlated with the coupling steps efficiency during the chain elongation.

## Result and Discussion

### Sequence

Caveolins are essential membrane structural proteins of caveolae where they act as scaffolding components also able to modulate the activity of sequestered signalling proteins [16]. Caveolin-1 has an unusual topology with the *N*- and *C*-terminal sequence in the cytosol. A topological model has been proposed based on the amino acid sequence analysis and on biochemical data. This model is composed of a hydrophobic domain (or intramembrane domain) flanked at both *N*- and *C*-termini by two amphipathic cytosolic segments (D82-R101) and (K135-I150) which interact with the cytoplasmic membrane interface. It should be noticed that the intramembrane domain most probably adopts an unusual hairpin structure within the membrane since its sequence (33 amino acids long) is too short to form two transmembrane segments. The D82-R101 sequence is also known as the scaffolding domain as it has been shown to interact with numerous signalling proteins. In order to get structural data at atomic level concerning the interaction of human caveolin-1 with the membrane interface, and carry on a preliminary CD and NMR study we intended to synthesise the D82-K135 fragment (Table 1) encompassing both the scaffolding (D82-R101) and intramembrane (L102-I134) domains [17]. Selectively $^{15}N$ labelled Ala and Gly residues as well as double $^{15}N$, $^{13}C$ labelled Phe99 were incorporated into the sequence. The Cys133 was replaced with a Ser to avoid the formation of disulfide bonds. In order to respect the global charge of this fragment in the whole protein, the target sequence (a) was designed with an acetylated *N*-terminus and an amide *C*-terminus.

### Synthesis and purification

The peptide was synthesized using conventional solid phase Fmoc/*tert*-butyl (*t*Bu) chemistry with a 433A peptide synthesizer on a 100 µmole scale [18]. Considering the swelling properties of PEG and PEG-Polystyrene based resins and their effectiveness for difficult sequences synthesis [19,20], we decided to choose a low-loading (0.23 mmole/g) NovaSyn®TGR resin (Merck chemicals Ntd) as solid support. This resin is derivatised with a modified Rink linker, which generates the carboxamide form of the peptide when subjected to TFA cleavage. We modified the standard 'FastMoc0.1Monitor Previous-Peak chemistry' of the manufacturer by extending 1-methyl-2-pyrrolidone (NMP) washing steps between the piperidine deliveries and introducing an additional NMP wash module after the coupling steps. 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (*N*-HATU)/diisopropylethylamine (DIPEA) were used as coupling reagents and Fmoc amino acid or pseudoproline dipeptides derivatives were coupled with a eightfold molar excess with regard to the resin. We did not introduce any postcoupling capping step with acetic anhydride. Fmoc removal was achieved by a 20% piperidine/NMP treatment. Conductive piperidine carbamate salts are generated as long as peptide-resin is not completely deprotected so that conductimetric monitoring of the synthesis can be set up by measuring salts release after each delivery of piperidine. According to the manufacturer protocol, a feedback allows to extend the deprotection step from three to five deliveries if conductivity of deprotection solution is within 5% of the previous value. For stability considerations, the NovaSyn®TGR resin is supplied with a free amino group, which explains that monitoring values for the first cycle did not exceed the background level (i.e. conductivity of the deprotection solution). The physicochemical behaviour of the growing peptide within the solvated matrix changes all along the synthesis, depending on the length and the composition of the amino acid sequence. Assuming that de-Fmoc monitoring reflects the reactivity of the growing *N*-terminus, each coupling difficulty could theoretically be inferred from the preliminary deprotection profile. At the end of the synthesis, acetylation of the free *N*-terminus moiety was performed with acetic anhydride before cleavage processing. The resin was then subjected to a 3-h treatment by a TFA cleavage mixture. The crude deprotected peptide was precipitated in cold diethylether and lyophilised. RP-medium pressure liquid chromatography (MPLC) purification was achieved by using C4-grafted Nucleoprep as packaging material and acetonitrile/propanol-2 (70/30) as organic eluent. Nevertheless, caveolin-1 (82–135) and truncated fragments were eluted from column at a notably high concentration (around 80%) of organic phase. Enriched or purified fractions were pooled and submitted to rotavapor-evaporation to reduce the organic solvent content, then diluted with water and freeze-dried. Crude and pre-purified peptidic mixtures were analysed by RP-HPLC and ESI-MS as described in the Material and Methods Section. Briefly, small amounts of the lyophilised materials were solubilised with 35% acetonitrile in 0.08% aqueous TFA to give a final concentration of 1.25 mg/ml. LC and LC-MS analysis were achieved by applying a linear gradient of acetonitrile/propanol-2 70/30 v/v in acidic aqueous buffer on a 300 Å C4 3.5 µm 2.1 × 100 mm Waters Symmetry column, with UV or MS detection.

### Effect of pseudoproline dipeptide positioning

In order to minimise the risk of aggregation during the synthesis, we took advantage of the presence in the sequence of Ser and Thr residues to integrate pseudoproline dipeptides, which have been proved to dramatically improve acylation and deprotection kinetics by disrupting the formation of secondary structures. In these dipeptides, Ser and Thr are reversibly protected as proline-like oxazolidine, and regenerated upon TFA cleavage [10]. We excluded to incorporate dipeptides in position Ser133 (no aggregation occurring beside the resin), Ser88 ($^{15}N$ labelled Ala87) and 91 [no commercial Thr–Thr($\psi^{Me,Me}$pro) available]. We thus inserted Leu–Ser($\psi^{Me,Me}$pro) dipeptides at Ser104 and Ser123 positions, which appear to be suitably distributed throughout the native sequence. Thr93 and Thr95 constituted a strategic alternative to incorporate a third pseudoproline dipeptide at an apart spaced position into the sequence. In a first attempt we chose to incorporate a Val–Thr($\psi^{Me,Me}$pro) at Thr95 position, which was the first occurring opportunity in the progress of the synthesis. The first synthesis was thus performed incorporating pseudoproline dipeptides at Ser123, Ser104 and Thr95 positions.

Conductivity monitoring trace of the synthesis (Figure 1(A)) showed difficult deprotection steps from Ile114 to Ile 109 cycles, auguring a series of difficult coupling steps. This is to be correlated with the interspace between the first pseudoproline dipeptide in position 123 and 114-109 region (>7 amino acid residues), where the inferred antiaggregative effect began to decrease. Surprisingly,



**Table 1.** Sequences of observed peptides and truncated fragments, where X represents a +42 *N*-terminal modification

| Peptide | Sequence |
|---|---|
| a | Ac-$^{82}$DGIWKASFTTFTVTKYWFYR**LLSALFGIPMALIWGIYFAILSFLHIWAVVPSI**K$^{135}$-$NH_2$ |
| b | H-$^{82}$DGIWKASFTTFTVTKYWFYR**LLSALFGIPMALIWGIYFAILSFLHIWAVVPSI**K$^{135}$-$NH_2$ |
| c | X-$^{89}$FTTFTVTKYWFYR**LLSALFGIPMALIWGIYFAILSFLHIWAVVPSI**K$^{135}$-$NH_2$ |
| d | X-$^{96}$KYWFYR**LLSALFGIPMALIWGIYFAILSFLHIWAVVPSI**K$^{135}$-$NH_2$ |
| e | X-$^{100}$YR**LLSALFGIPMALIWGIYFAILSFLHIWAVVPSI**K$^{135}$-$NH_2$ |

The intramembrane domain is shown in bold.

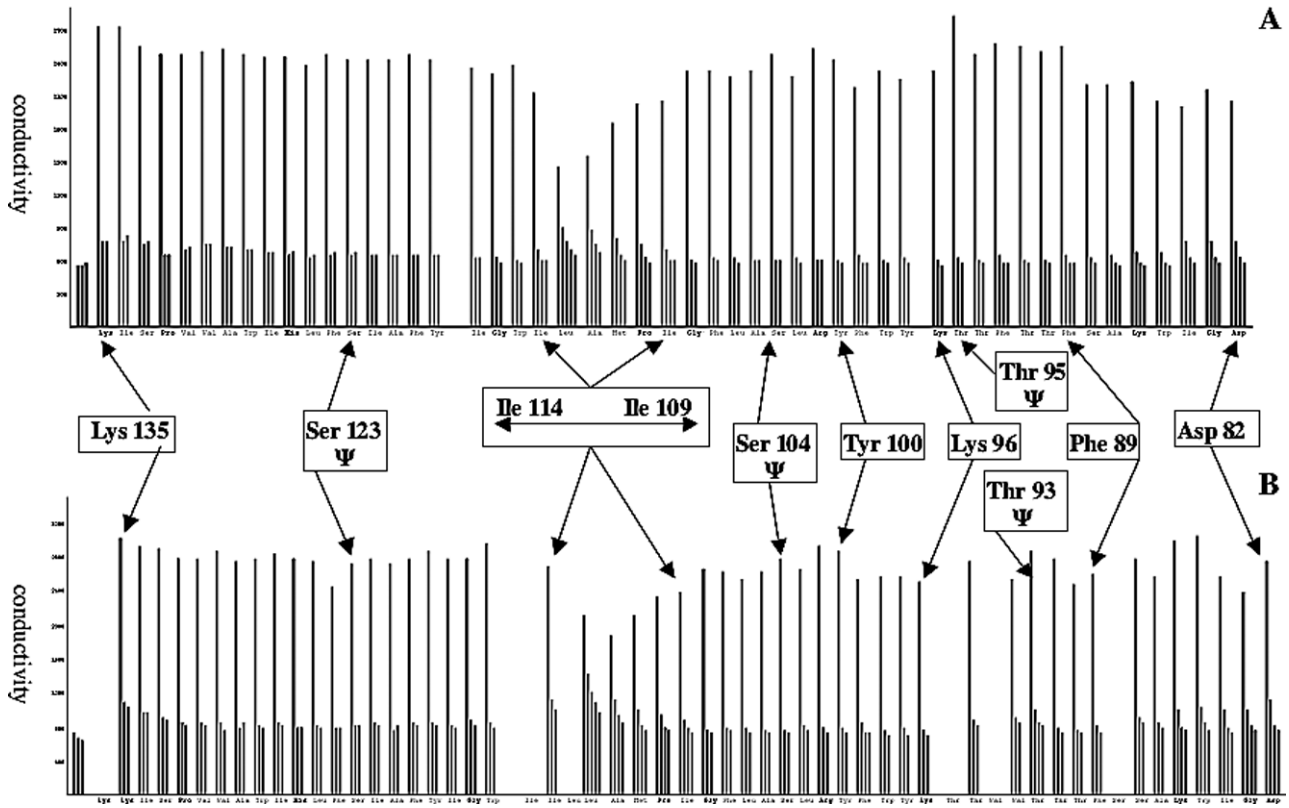



**Figure 1.** Conductivity monitoring traces of Fmoc deprotection for both D82-K135 caveolin peptide synthesis 1 (A) & 2 (B).

MS analysis of the crude product obtained after synthesis, *N*-terminus final acetylation and cleavage from the resin (Figure 2(A)) attested the presence of three major truncated fragments (Table 1: c, d, e) longer than those expected referring to the monitoring profile. Furthermore, when analysing the *not acetylated* crude product, we showed that those fragments, i.e. (c): observed mass 5600.2 (theor 5558.7); (d): observed mass 4802.2 (theor 4760.8) and (e): observed mass 4167.9 (theor 4126.0), presented a +42 Da mass deviation from expected values (Figure 2(B)). On the contrary, the experimental mass observed for the free amine target peptide (b) (observed mass 6318.0) corresponded to the theoretical MW (6318.5). This suggests that a competitive side reaction occurred during the coupling step, leading to a +42 capping adduct. The ratio of +42 fragment appeared to reflect the coupling difficulty and therefore the aggregation state of the chain. The analytical HPLC profile of the *not acetylated* crude mixture distinguishes the target peptide from the main truncated fragments (Figure 3).

Unfortunately, the fragment (c) coeluted with the *acetylated* target peptide (a) in our purification conditions. Considering the poor coupling yield of Ser88 and the difficulty to separate the target peptide (a) from the truncated fragment (c) by RP-HPLC, we tried to improve the synthesis by modifying the strategy of pseudoproline dipeptides incorporation. We started a second synthesis with a Phe–Thr($\psi^{Me,Me}$pro) inserted at Thr93 position instead of Val–Thr($\psi^{Me,Me}$pro) at Thr95. This shift reduced the space between peudoproline dipeptide and Phe89 from 5 to 3 residues. Therefore, we expected the antiaggregative effect would be maintained until the difficult Ser88 coupling proceeds. Besides, we introduced six double coupling cycles at positions selected with regard to the monitoring profile of the first synthesis and after identification of truncated peptides, with the exception of the labelled amino acids. The resultant monitoring profile was slightly improved from the deprotection of Ser88 up to the end (Figure 1(B)). After synthesis and cleavage from the resin, the crude peptide was entirely recovered by precipitation in diethylether, unlike the first synthesis crude mixture, which remained partially in the residual diethylether phase and was recovered up to half by extraction with water as solid material



Journal of PeptideScience

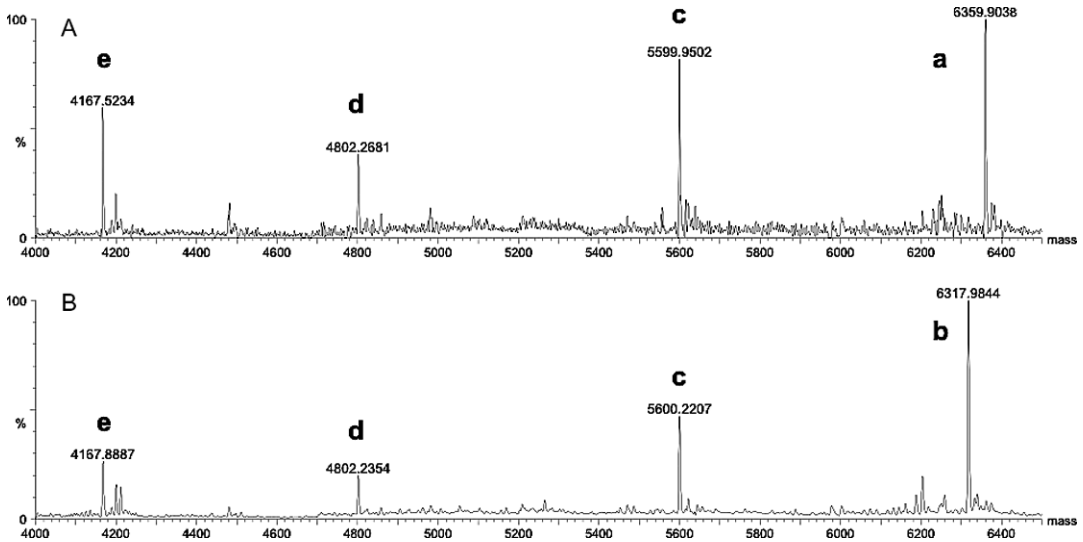



**Figure 2.** MaxEnt 1 deconvoluted ESI-MS spectra of acetylated (A) or not-acetylated (B) crude caveolin peptide (synthesis 1). a: Ac-D82-K135, b: H-D82-K135, c: X-F89-K135, d: X-K96-K135, e: X-Y100-K135 where X = +42 *N*-ter modification.

at the diethylether/water interface. This is to be correlated with the notably improved elution profile of the crude peptide, which was remarkable by the decrease of the previous hydrophobic truncated peptides (Figure 4(A)). Considering the capping side reaction occurring on the low-reactive *N*-terminus of the peptidic chain, it seems unlikely that Ser88 coupling improvement might be the exclusive result of a scheduled double coupling cycle. Slightly modifying the pseudoproline dipeptide incorporation position might have favourably shifted the structure-disrupting effect so that improved Ser88 coupling kinetic prevented the capping side reaction. Avoiding the formation of the truncated fragment (c), the acetylated target peptide could be isolated easily as described in the Material and Method Section, with a total yield of 3%. Purity was checked by RP-HPLC (Figure 4(B)). The peptide was finally characterized by ESI-MS. The experimental data (6359.8711) was consistent with the expected mass (6360.546) (Figure 5).

## Capping side-reaction

As previously described, difficult coupling reactions generated *N*-capped truncated peptides, for which the observed modifications, compared with the free amine sequences, resulted to a mass increase of +42 Da. This side reaction is mostly undetectable when scheduling a systematic postcoupling capping reaction with acetic anhydride, which lead to truncated peptides presenting a similar +42 adduct. On one hand, considering a possible acetylation occuring in the presence of acetic acid, we checked for a possible contamination the amino acid derivatives Fmoc–Ser(tBu)–OH, Fmoc–Val–Thr($\psi^{Me,Me}$pro)–OH and $^{13}$C-,$^{15}$N-labelled Fmoc–Phe–OH, which were implicated in the poor coupling steps of Ser88, Thr95 and Phe99 in the first synthesis and the resulting generation of the three major truncated fragments (c), (d) and (e), respectively. As a matter of fact, 1 H NMR data showed complete absence or negligible presence of acetic acid contamination. On the other hand, we observed in the 1 H NMR spectrum of the truncated fragment (c), isolated from the not acetylated crude peptide and solubilised in TFE-d2OH, two signals at 2.70 and 2.71 ppm (see Supporting Information). Those signals were not observed in the whole purified acetylated target peptide (a), for which a singlet recorded in the same conditions at 2.15 ppm was attributed to the acetyl protons (data not shown).

From these observations and considering the occurrence of the postulated side reaction during the coupling step of the synthesis, we suspected the implication of HATU, which was already well known to react in a competitive way with peptide *N*-terminus to yield truncated peptides with a tetramethyl guanidino termination by-product with a $\Delta m = +98$ [21,22]. Moreover, it is well established that the guanidinium salt of HATU, which is commercially supplied, is a less efficient coupling reagent than the uronium isomer [23]. We suggest that the +42 adduct observed upon side termination of the synthesis could

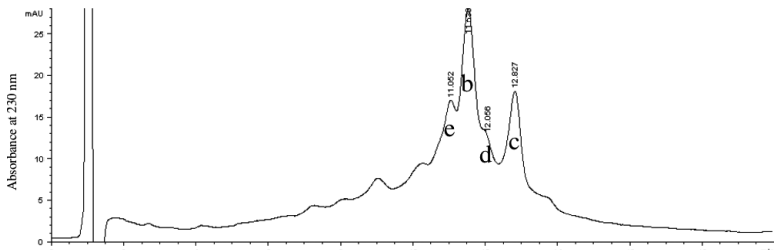



**Figure 3.** Analytical HPLC profile of the not acetylated crude peptide (synthesis 1). b: H-D82-K135, c: X-F89-K135, d: X-K96-K135, e: X-Y100-K135 (Waters Symmetry 300 C4 3.5 µm 2.1 × 100 mm column; 75–100%B in 20 min, 0.35 ml/min; A: 0.08%TFA aq; B: MeCN/ipropOH 70/30; 230 nm UV detection; LC-MS data not shown).



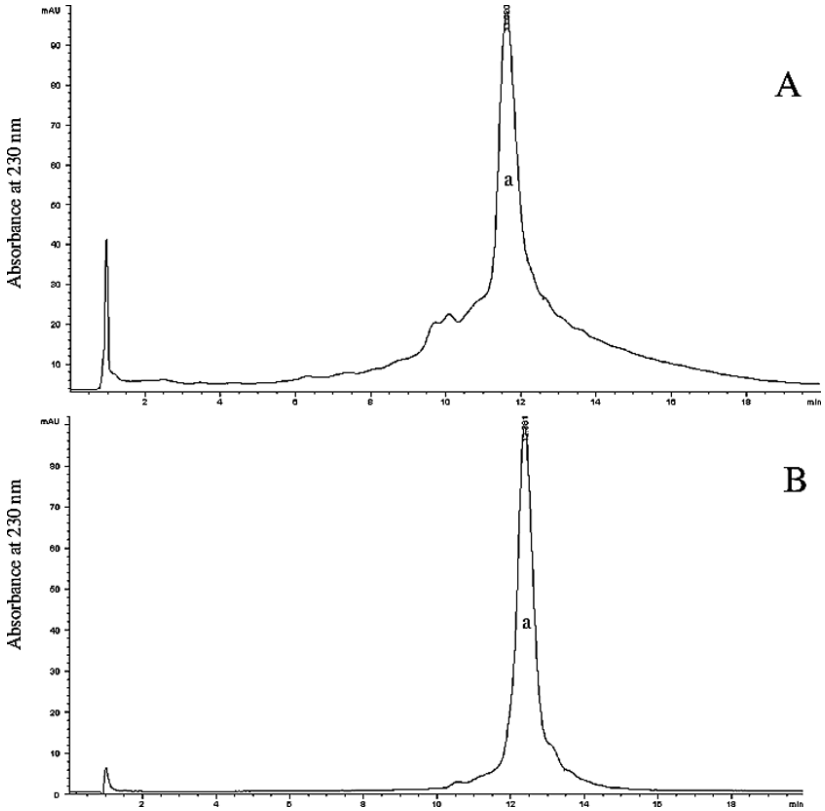



**Figure 4.** Analytical HPLC profiles of crude (A) and purified (B) acetylated D82-K135 caveolin peptide (synthesis 2). a: Ac-D82-K135 (Waters Symmetry 300 C4 3.5 µm 2.1 × 100 mm column; 75–100% B in 20 min, 0.35 ml/min; A: 0.08% TFA aq; B: MeCN/ipropOH 70/30; crude was solubilised in aqTFA/TFE 80 : 20 v/v; purified peptide was directly loaded after evaporation of the elution mixture).

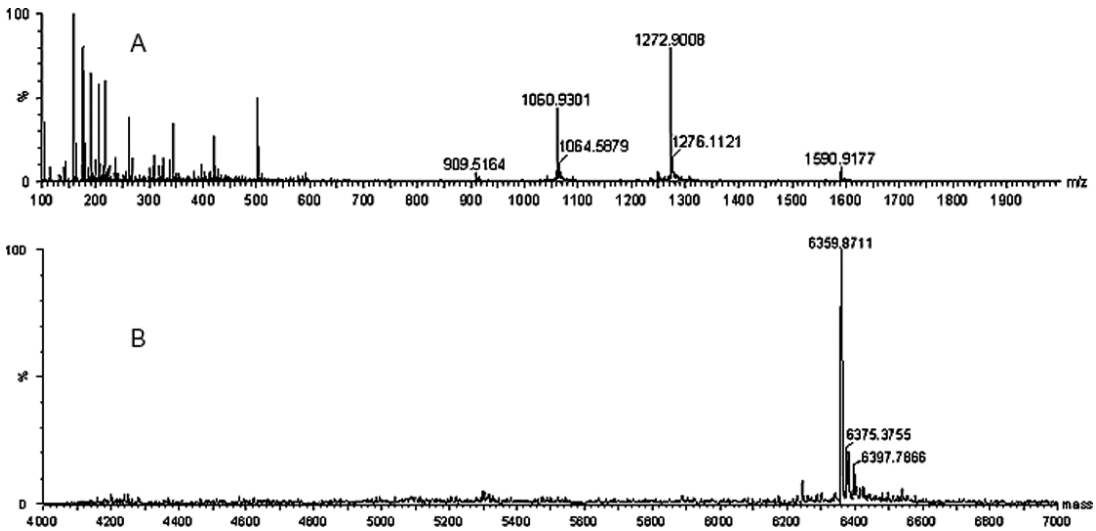



**Figure 5.** Raw (A) and MaxEnt 1 deconvoluted spectra (B) of purified acetylated D82-K135 caveolin peptide.

be assigned to a dimethyldiazenium *N*-terminal cap, which could be in agreement with the 1 H-NMR data. In order to confirm the chemical stability of the guanidino derivatives [21] and to reject a possible two steps mechanism involving the conversion upon TFA cleavage of the +98 modified fragments, we generated the +98 *N*-capped tripeptide GGK by overloading the free amine peptidyl-resin with HATU/DMF solution and we submit the resulting resin to the cleavage procedure previously described for the caveolin peptide. As shown by mass spectrometry of the crude (observed mass 358.19, theor 358.25) and 1 H NMR analysis of the purified peptide (12 dimethylaminoprotons giving rise to a signal near 3.0 ppm), the +98 adduct remained stable in our cleavage conditions. In the absence of a +42 modified tripeptide, we were able to exclude the implication of TFA cleavage in the mechanism of +42 modified truncated peptides generation. Correspondingly, the chemical nature of the side product remains elusive and requires additional investigations for rational explanations.



Journal of PeptideScience

## Material and Methods

### Peptide synthesis

Synthesis was carried out on an ABI 433 synthesizer (Applied Biosystems, Foster City, CA, USA) equipped with a conductivity flow cell to monitor Fmoc deprotection. NovaSyn® TGR resin and pseudoproline (oxazolidine) dipeptides were purchased from Merck-Novabiochem (Darmstadt, Germany). L-alanine *N*-Fmoc $^{15}$N (98% enrichment) and L-phenylalanine U-$^{13}$C9 (98% enrichment) $^{15}$N (98% enrichment) were from Eurisotop (Saint-Aubin, France). Standard Fmoc amino acids were obtained from Applied Biosystems, and side-protected as followed : *t*Bu for aspartic acid, serine, threonine and tyrosine, trityl for histidine, Boc for lysine and tryptophan, and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl for arginine. Fmoc-amino acids and pseudoproline dipeptides were activated with HATU/DIPEA and single coupled with a eightfold molar excess with regard to the resin. Double coupling cycles were applied over the second synthesis to residues Ser88, Val94, Thr95, Leu113, Ile114 and Lys135. Both coupling reagents, as well as *N*-methyl pyrrolidone (NMP), were purchased from Applied Biosystems. Piperidine was purchased from Sigma-Aldrich (St Louis, MO, USA). Specific *N*-terminal acetylation was achieved manually at the end of the synthesis by stirring the peptide resin for 30 min in an excess of an acetylation cocktail containing 10% acetic anhydride in DMF.

### Cleavage from the resin

Cleavage from the solid support and deprotection of the amino acid side chains were accomplished in one step by treatment with 92.5 : 2.5 : 2.5 : 2.5 mixture of TFA Applied Biosystems, ethanedithiol, triisopropylsilane (Sigma-Aldrich) and water for 3 h at room temperature. After filtration of the resin, the cleavage mixture was poured into ice-cold diethyl ether. The peptide was then recovered by filtration of the precipitate, redisolved in a mixture of TFA 0.08% and acetonitrile and lyophilised.

### HPLC analysis

Analysis of crude mixtures and purity control of the final peptides were performed by RP-HPLC on an Agilent (Santa Clara, CA, USA) 1100 Series liquid chromatograph and monitored with a photodiode array detector by absorbance at 230 nm. A linear gradient of 75–100% solvent B (solution of acetonitrile and isopropanol 7 : 3 v/v) in solvent A (0.08% aqueous TFA) over 20 min (1.25% B/min) was applied at a 0.35 ml/min flow rate on a Symmetry300 C4 3.5 µm 2.1 × 100 mm column (Waters, Manchester, UK). LC-MS data were obtained using a Waters Alliance 2695 system comprising a 2487 dual absorbance detector and coupled with a TOF-MS detector (Waters Q-TOF Micro). The same gradient was applied on the same column with the following eluents: A: water containing 0.05 formic acid and 0.04% TFA, B: solution of acetonitrile and isopropanol 7 : 3 v/v containing 0.025% formic acid. Data acquisition and process are described bellow.

### Purification

Solubilisation of quantitative amounts of crude peptides was achieved by mixing the lyophilised material in TFE. The solutions were diluted with TFA 0.08% and sonicated so that the final concentrations were 3 mg/ml of peptide in aqTFA/TFE 80 : 20 v/v. This material (loading 60 mg per run) was directly purified by RP-MPLC (AP-100/200 flash, Armen Instrument, Saint Ave, France) on a preparative column (26 × 313 mm) packed with 300 Å 20 µm C4 Nucleoprep packing (Macherey & Nagel GmbH & Co, Düren, Germany), by applying a linear gradient (1%/min) of 40–100% solvent B (solution of acetonitrile and propanol-2, 7 : 3 v/v) in solvent A (0.08% aqueous TFA) over 60 min at a 20 ml/min flow rate. The purification was monitored at 214 nm (UV detector K2501, Knauer, Berlin, Germany). Suitable fractions were pooled and evaporated (Rotavapor), then diluted with water and lyophilised. A second step of purification was necessary, using the same protocol, by loading 40 mg of target peptide enriched mixture per run. The exact concentration of the purified peptide was determined by quantitative Amino Acid Analysis (total yield: 3%).

### Electrospray ionisation mass spectrometry

Mass spectrometry was carried out on a quadrupole-TOF Micro mass spectrometer (Waters) equipped with a Z-spray API source and calibrated with a phosphoric acid calibration solution. Capillary, sample cone and extraction cone voltages were set at 3 kV, 40 V and 10 V, respectively. Source and desolvation temperatures were set at 80 and 250 °C, respectively (raised to 120 and 400 °C in the higher flow rate conditions of LC). Data were acquired by scanning over the m/z range 150–2000 at a scan rate of 1 s and an interscan delay of 0.1 s. Lyophilised crude and purified products were dissolved in a mixture of water/methanol/acetic acid 49.5/49.5/1 v/v/v at a concentration of 1 µg/µl and analysed in positive-ion mode by infusion at a flow rate of 5 µl/min. Three hundred spectra were combined and the resultant raw multicharged spectra were processed using the MaxEnt 1 deconvolution algorithm embedded in the Masslynx software. LC/MS data were obtained by selecting and combining spectra of separate peaks and shoulders of the Total Ionic Current chromatograms.

### NMR experiments

The lyophilized samples were solubilized at a concentration of about 1.5 mM in 100% TFE-d2 (2,2,2-trifluoroethyl-1,1-$d_2$ alcohol >99% isotopic enrichment – Eurisotop). NMR experiments were performed at 20 °C on a DRX spectrometer (Bruker Daltonic GmbH, Bremen) operating at 600 MHz 1 H frequency equipped with a cryoprobe. Chemical shifts were referenced with respect to the residual methylene protons of TFE appearing at 3.98 ppm.

## Conclusions

We described the synthesis of a 54 amino acid long peptide encompassing a 33 hydrophobic amino acid sequence using pseudoproline dipeptides. Without any acetylation during the synthesis, we report a capping side reaction yielding truncated peptides with a +42 adduct. The purification of the target peptide was successful by lowering the formation of those +42 truncated fragments, which tend to coeluate in conventional RP-HPLC procedures, thanks to the considered incorporation of pseudoproline dipeptides during the synthesis. In the current synthesis, pseudoproline dipeptides have been shown to represent a useful and essential improvement to carry out difficult amino acid sequence synthesis. By showing the effect on the synthesis yield of a slight shift in their positioning, our results aim to highlight the impact of the pseudoproline dipeptide incorporation strategy.



### Supporting information

Supporting information may be found in the online version of this article.

## References

1 Josic D, Clifton JG, Kovac S, Hixson DC. Membrane proteins as diagnostic biomarkers and targets for new therapies. *Curr. Opin. Mol. Ther.* 2008; **10**: 116–123.
2 Hopkins AL, Groom CR. The druggable genome. *Nat. Rev. Drug. Discov.* 2002; **1**: 727–730.
3 Tan S, Tan HT, Chung MC. Membrane proteins and membrane proteomics. *Proteomics* 2008; **8**: 3924–3932.
4 Johnson EC, Kent SB. Studies on the insolubility of a transmembrane peptide from signal peptide peptidase. *J. Am. Chem. Soc.* 2006; **128**: 7140–7141.
5 Khemtemourian L, Lavielle S, Bathany K, Schmitter JM, Dufourc EJ. Revisited and large-scale synthesis and purification of the mutated and wild type neu/erbB-2 membrane-spanning segment. *J. Pept. Sci.* 2006; **12**: 361–368.
6 Coïc YM, Vincent M, Gallay J, Baleux F, Mousson F, Beswick V, Neumann JM, de Foresta B. Single-spanning membrane protein insertion in membrane mimetic systems: role and localization of aromatic residues. *Eur. Biophys. J.* 2005; **35**: 27–39.
7 Clayton D, Shapovalov G, Maurer JA, Dougherty DA, Lester HA, Kochendoerfer GG. Total chemical synthesis and electrophysiological characterization of mechanosensitive channels from *Escherichia coli* and *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 2004; **101**: 4764–4769.
8 Tiburu EK, Dave PC, Vanlerberghe JF, Cardon TB, Minto RE, Lorigan GA. An improved synthetic and purification procedure for the hydrophobic segment of the transmembrane peptide phospholamban. *Anal. Biochem.* 2003; **318**: 146–151.
9 Sato T, Kawakami T, Akaji K, Konishi H, Mochizuki K, Fujiwara T, Akutsu H, Aimoto S. Synthesis of a membrane protein with two transmembrane regions. *J. Pept. Sci.* 2002; **8**: 172–180.
10 Wohr T, Wahl F, Nefzi A, Rohwedder B, Sato T, Sun XC, Mutter M. Pseudo-prolines as a solubilizing, structure-disrupting protection technique in peptide synthesis. *J. Am. Chem. Soc.* 1996; **118**: 9218–9227.
11 Coin I, Beyermann M, Bienert M. Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nat. Protoc.* 2007; **2**: 3247–3256.
12 Cremer GA, Tariq H, Delmas AF. Combining a polar resin and a pseudo-proline to optimize the solid-phase synthesis of a 'difficult sequence'. *J. Pept. Sci.* 2006; **12**: 437–442.
13 White P, Keyte JW, Bailey K, Bloomberg G. Expediting the Fmoc solid phase synthesis of long peptides through the application of dimethyloxazolidine dipeptides. *J. Pept. Sci.* 2004; **10**: 18–26.
14 Goncalves V, Gautier B, Huguenot F, Leproux P, Garbay C, Vidal M, Inguimbert N. Total chemical synthesis of the D2 domain of human VEGF receptor 1. *J. Pept. Sci.* 2009; **15**: 417–422.
15 Sampson WR, Patsourias H, Ede NJ. The synthesis of 'difficult' peptides using 2-Hydroxy-4-Methoxybenzyl or pseudoproline amino acid building blocks: a comparative study. *J. Pept. Sci.* 1999; **5**: 403–409.
16 Cohen AW, Hnasko R, Schubert W, Lisanti MP. Role of caveolae and caveolins in health and disease. *Physiol. Rev.* 2004; **84**: 1341–1379.
17 Le Lan C, Neumann JM, Jamin N. Role of the membrane interface on the conformation of the caveolin scaffolding domain: a CD and NMR study. *FEBS Lett.* 2006; **580**: 5301–5305.
18 Chan WC, White PD. *Fmoc Solid Phase Peptide Synthesis. A Practical Approach*. Oxford University Press: London, 2000.
19 Garcia-Martin F, White P, Steinauer R, Cote S, Tulla-Puche J, Albericio F. The synergy of ChemMatrix resin and pseudoproline building blocks renders RANTES, a complex aggregated chemokine. *Biopolymers* 2006; **84**: 566–575.
20 Kates SA, McGuinness BF, Blackburn C, Griffin GW, Sole NA, Barany G, Albericio F. "High-load" polyethylene glycol-polystyrene (PEG-PS) graft supports for solid-phase synthesis. *Biopolymers* 1998; **47**: 365–380.
21 Gausepohl H, Pieles U, Frank R. Schiff base analog formation during in situ activation by HBTU & TBTU. *Peptides, Proceedings of the 12 American Peptide Symposium*, Smith JA, Rivier JE, (eds). ESCOM: Leiden, 1992; 523–524.
22 Albericio F, Bofill JM, El-Faham A, Kates SA. Use of onium salt-based coupling reagents in peptide synthesis. *J. Org. Chem.* 1998; **63**: 9678–9683.
23 Carpino LA, Imazumi H, El-Faham A, Ferrer FJ, Zhang C, Lee Y, Foxman BM, Henklein P, Hanay C, Mugge C, Wenschuh H, Klose J, Beyermann M, Bienert M. The uronium/guanidinium Peptide coupling reagents: finally the true uronium salts. *Angew. Chem. Int. Ed. Engl.* 2002; **41**: 441–445.

