Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 437–442
Published online 23 January 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.746

JOURNAL OF PEPTIDE SCIENCE

Combining a polar resin and a pseudo-proline to optimize the solid-phase synthesis of a 'difficult sequence'

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Received 7 November 2005; Revised 29 November 2005; Accepted 4 December 2005

Abstract: This paper describes the optimization of a synthesis of a difficult sequence related to a 12-mer sequence of a Pan DR epitope (PADRE). Elongation was followed by on-line monitoring of the N^{α}-Fmoc removal adapted for the batch methodology. Studying the intrinsic factors related to the peptide-resin, such as substitution level, resin nature and backbone protecting group, has led to an increase in the elongation yield and purity of the crude peptide. Optimal elongation was obtained by combining a polar resin such as PEGA and a pseudo-proline as the backbone protecting group. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide synthesis; difficult sequence; polar resin; pseudo-proline; padre epitope; on-line UV monitoring

INTRODUCTION

Therapeutic vaccines comprising defined antigens used for the development of antitumor vaccination strategies has led to a renewed interest in the immunological applications of synthetic peptides [1,2]. For efficient stimulation of antitumor immune responses, the addition of a T-helper epitope such as the universal Pan HLA DR-binding epitope (PADRE, [3]), is crucial [4]. In our ongoing program aimed at synthesizing chemically defined antitumoral antigens to mimic the tumor-associated form of the MUC1 protein [5], solid-phase synthesis of the Pan DR epitope (PADRE) epitope has proven to be difficult. By obtaining these artificial antigens as homogenous as possible, the synthesis of the PADRE epitope should be optimized.

Ever since the pioneering work of Merrifield, the solid-phase technique has revolutionized peptide syntheses. Polymer supports [6,7], protecting groups [8] and coupling reagents [9] have become the subject of considerable interest to enable the routine synthesis of peptides to approximately 50 amino acids in length [10]. Despite all these improvements, problems still do occur, characterized by a sharp decrease in the coupling yields of successive amino acids accompanied by a change in the resin structure with loss of the gel-like character and the appearance of a macroscopic shrinking of the solid support. These problems inherent in the so-called 'difficult sequence' represent the most serious drawback in stepwise syntheses [11,12] leading to low elongation yields and nonhomogenous crude peptides. They can be attributed to peptide aggregation mediated by hydrophobic effects or hydrogen bonding. Hence, secondary structures such as β-sheets are stabilized leading to an ineffective solvation of either the peptide chain or the polymeric matrix.

Many solutions proposed to overcome these problems and make some of the difficult sequences easier include external factors of the peptide-resin such as the use of different solvents, chaotropic salts, and elevated temperatures during the coupling step [13]. However, intrinsic factors, i.e. factors related to either the peptide-resin, the peptide chain or the resin, seem to be generally better at preventing the hydrogen-bonded association of the peptide chain. Diminishing the loading of the resin has improved the synthesis of the difficult sequence [14] as well as the use of a solubilizing protecting group, an amide-protecting group within the peptide chain [15,16], the $O \rightarrow N$ shift method [17,18] and a presequence [19]. Polar matrices whose swelling characteristics are believed to favor solvation and the random coil conformation of the growing peptide chains, have improved the synthesis of 'difficult sequences' [20–22].

Here, we compare several intrinsic factors compatible with standard protocols for the Fmoc strategy to improve the solid-phase synthesis of the 12-mer PADRE epitope including a resin with low loading, commercially available polar matrices and an amide-protecting group. These results were also applied to a longer chimeric peptide containing the PADRE difficult sequence.

RESULTS AND DISCUSSION

PADRE Epitope as a 'Difficult Sequence'

Elongation was carried out by batch methodology on an automated synthesizer using Fmoc/*t*Bu chemistry

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[23]. As a solvent, NMP was preferred over DMF to overcome interchain interactions and to improve the coupling efficiency [14]. Single coupling was performed with a 10-fold excess of N^{α}-Fmoc amino acid and HBTU [24]/DIEA as coupling reagents. HOBt, whose addition was shown to increase the coupling yield in the presence of an onium coupling reagent, was included in the reaction mixture [25]. The coupling step was systematically followed by a capping step with Ac_2O. Fmoc was removed by three piperidine treatments and the fluorenylmethyl piperidine adduct was recorded on-line by UV spectroscopy [26]. When the third piperidine deprotection afforded an absorbance data of 3% compared to the first one, up to five conditional piperidine treatments were carried out. This is characteristic of a sluggish and incomplete Fmoc removal and indicates the onset of the internal aggregation of the peptide in a resin environment [26,27]. The crude peptide released after final TFA treatment was analyzed by HPLC and mass spectrometry.

The elongation of the PADRE epitope was first run on a conventional resin, i.e. polystyrene cross-linked with 1% divinylbenzene (DVB-PS), loaded with a Rink-amide linker at a 0.76 mmol/g substitution. This moderate level of substitution is routinely used for the synthesis of 10–15 mer peptides. Under these conditions, the UV deprotection trace showed incomplete aminoacylation for the last five residues (Figure 1(A)) accompanied with a sluggish deprotection of the Fmoc group. Double coupling of the last five residues partly improved the synthesis but not enough for a satisfactory elongation yield (not shown). Furthermore, the resin recovered at the end of the elongation had obviously shrunk, having lost its gel-like appearance to a granular one. Together, these data clearly show that the PADRE epitope is a 'difficult sequence'. Further improvement of the solid-phase synthesis was made, keeping in mind an easily and widely usable methodology on an automated synthesizer.

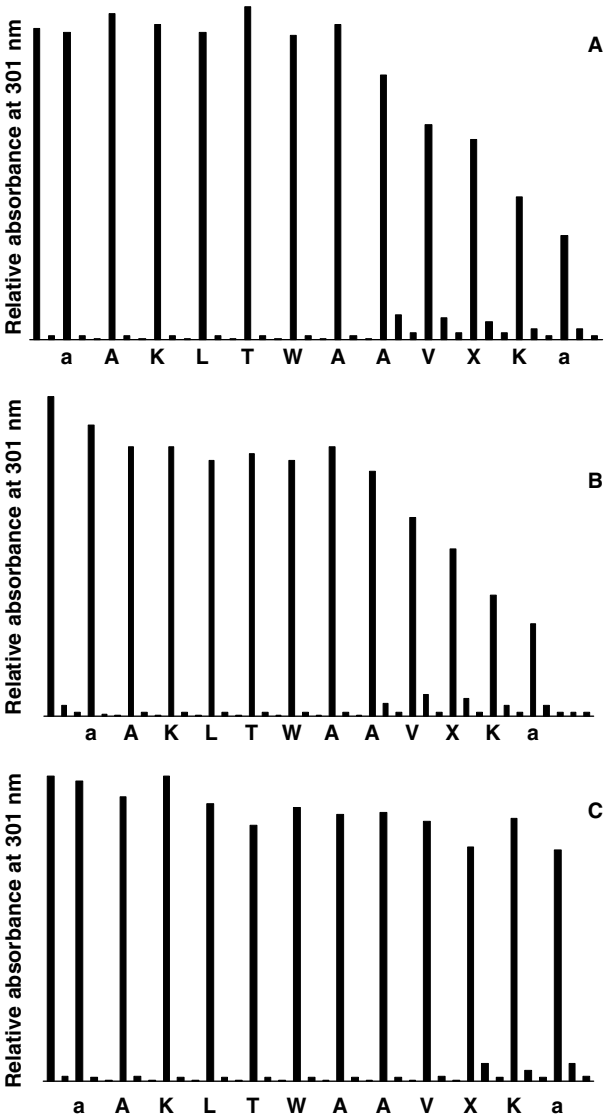

Figure 1 UV N^{α}-Fmoc deprotection trace during the elongation of the PADRE epitope on a DVB-PS resin (A) at a 0.76 mmol/g substitution; elongation yield: 37%, (B) at a 0.4 mmol/g substitution; elongation yield: 45%; purity of the crude peptide: 73% (C) at a 0.26 mmol/g substitution; elongation yield: 82%; purity of the crude peptide: 81%.

Influence of the Loading

Besides the 0.76 mmol/g substitution, two lower values were tested (Figure 1). A loading of 0.4 mmol/g resulted in 45% elongation yield only. As expected, the mass spectrometry analysis of the HPLC chromatogram (Figure 2) revealed truncated peptides (Table 1). The low loading of 0.23 mmol/g afforded an elongation yield of 82% with good purity (81%). These results confirm that the coupling rates resulting from a peptide aggregation were obvious at a high resin substitution and were improved at low loading.

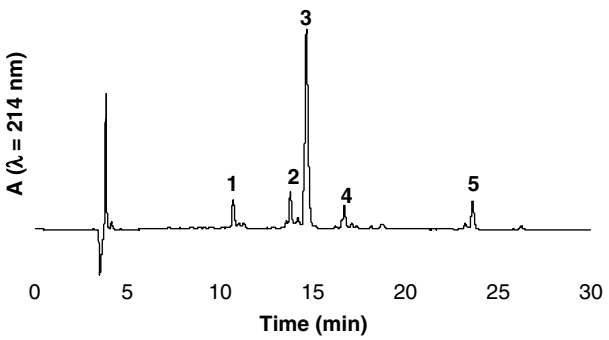

Figure 2 Analytical HPLC profile of the crude PADRE peptide after synthesis on a PS-resin substituted at 0.4 mmol/g.

Use of a Backbone Protecting Group

The presence of the imino acid proline residues in a sequence has been shown to improve the elongation yield [28] probably because the hydrogen bond for-

Table 1 Mass spectrometry analysis after analytical HPLC of the PADRE sequence synthesized on a PS-resin substituted at 0.4 mmol/g

Peak number	Purity[a]	M found[b]	Attributed to	M calculated
1	6.5	871	*Ac*-AAWTLKAa-*NH*$_2$	$C_{41}H_{65}N_{11}O_{10}$ 871.49
2	9	970	*Ac*-VAAWTLKAa-*NH*$_2$	$C_{46}H_{74}N_{12}O_{11}$ 970.56
3	73	1280.91	*H*-aKXVAAWTLKAa-*NH*$_2$	$C_{62}H_{104}N_{16}O_{13}$ 1280.80
4	5	1386	?	
5	6.5	1123.69	*Ac*-XVAAWTLKAa-*NH*$_2$	$C_{55}H_{89}N_{13}O_{12}$ 1123.68

[a] Estimated by integration of HPLC peaks at 214 nm.
[b] Monoisotopic mass determined by MALDI-TOF.
? Not attributed

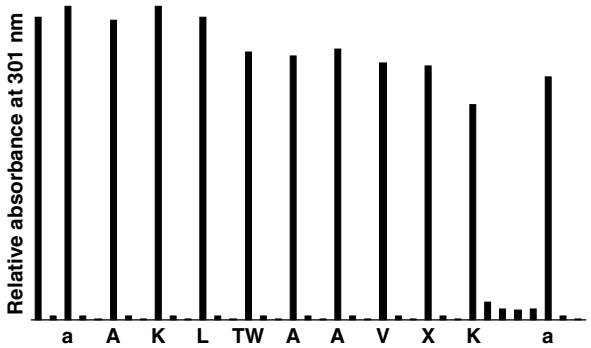

Figure 3 UV N^{α}-Fmoc deprotection trace during the elongation of the PADRE epitope on a DVB-PS resin at 0.4 mmol/g substitution, using a pseudo-proline for the introduction of the Thr-Trp dipeptide. Elongation yield: 89%; purity of the crude peptide: 87%.

mation was avoided. To incorporate a high proportion of a tertiary amide, 2-hydroxy-4-methoxybenzyl (Hmb) has been developed as an amide-protecting group [16] and has been used in the efficient syntheses of difficult sequences [12]. An alternative method consists in incorporating Ser/Thr as dimethyloxazolidine analogs, the so-called pseudo-prolines [15]. When comparative studies were undertaken [29,30], Hmb backbone protection was found to be inferior to pseudo-proline. As the PADRE epitope contains a threonyl residue before the difficult sequence, the pseudo-dipeptide Fmoc-Trp(Boc)-Thr($\psi^{Me,Me}$ pro)-OH was used with an elongation carried out on a PS-resin substituted at 0.4 mmol/g. When compared to the elongation carried out without pseudo-proline (Figure 1(B)), the UV trace revealed a great improvement in the elongation yield (Figure 3), which reached 89% with satisfactory purity of the crude peptide (87%). Nevertheless, a slight drop in the UV monitoring was observed after deprotection of the dipeptide building block and sluggish N^{α}-deprotection of the lysyl residue was still observed.

Nature of the Polymeric Matrix

Polar polymeric supports have proved to be beneficial for the synthesis of difficult sequences such as acyl carrier protein ACP-65-74 [21,32] or other hydrophobic sequences [22]. This is consistent with the role of cosolvent attributed to polymeric supports [31]. Among the polar resins containing polyethyleneglycol (PEG), two commercially available matrices were used for synthesizing the PADRE epitope: one grafted PEG-PS, the Tentagel resin [32] and one based on cross-linked PEG, the poly(ethylene glycol)-poly(acrylamide) (PEGA) resin [30].

Fmoc-Rink-amide linker was manually coupled to aminomethyl Tentagel or PEGA resins using the *in situ* protocol described for Fmoc-chemistry [34]. The completion of the reaction was checked by the Kaiser test. The original loading determined by UV measurement of the fluorenylmethylpiperidine adduct was 0.26 and 0.36 mmol/g for the Tentagel and PEGA resins, respectively. The use of Tentagel and PEGA resins gave similar results for the elongation yield and purity of the crude peptide (Figure 4). The UV monitoring traces indicate an impressive improvement in the elongation of the PADRE epitope on the PEGA resin (Figure 4(B)) over the PS-resin (Figure 1(B)) at a similar loading. It is difficult to assess the use of Tentagel (Figure 4(A)) because the loading at which it was tested was the same as that which gave satisfactory results on the PS-resin (Figure 1(C)). Nevertheless, a brief study comparing a grafted PEG-PS resin *versus* a PEGA resin for continuous flow synthesis has reported the latter as being more efficient [35].

Implications for the Synthesis of a Longer Peptide Containing the PADRE Epitope

PADREmuc: H-aKXVAAW^{7}T^{8}LKAaPPAHGVTSAPDTRPAPGSTAKA-NH-NH$_2$. The PADRE epitope is an excellent 'difficult sequence' model for comparing the different solutions advocated for improving the solid-phase synthesis. Among the solutions tested, the PS-resin at a loading of 0.23 mmol/g or the PS-resin at a loading of 0.4 mmol/g with the use of a pseudo-proline dipeptide or polar resin at a loading of 0.36 mmol/g gave similar data, satisfactory with respect to the elongation yield and purity of the crude peptide. Nevertheless,

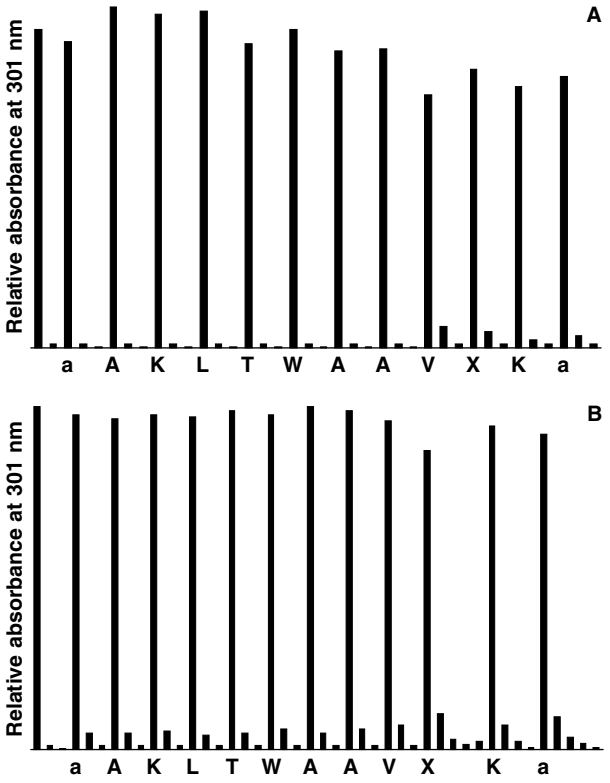

Figure 4 UV N^{α}-Fmoc deprotection trace during the elongation of the PADRE epitope (A) on a tentagel resin at a 0.26 mmol/g substitution, elongation yield: 91%; purity of the crude peptide: 93%; (B) on a PEGA resin at a 0.36 mmol/g substitution; elongation yield: 87%; purity of the crude peptide: 90%.

none of these solutions completely avoided some of the sluggish N^{α}-Fmoc deprotections revealed by additional piperidine treatments. Combining a polar resin such as a PEGA resin with the use of dipeptide pseudo-proline should improve the solid-phase synthesis and prevent sluggish N^{α}-Fmoc deprotections. This hypothesis was tested with the elongation of a 34-mer chimeric peptide composed of the PADRE epitope colinearly synthesized at the *N*-terminus of the MUC1 peptide (PPAHGVTSAPDTRPAPGSTAKA) to take advantage of the presequence effect [19]. PADRE-Muc was obtained with a hydrazide function at the *C*-terminus for further use in hydrazone ligation. Elongation was carried out on an aminomethyl PEGA resin substituted with Boc-Ala-4-(oxymethyl)phenylacetic acid to form the phenylacetamidomethyl (PAM) linker [5]. The elongation yield was good (86%) but there was a drop in the on-line UV trace from the Thr^{8} of the PADRE sequence (Figure 5(A)). Combining the use of a PEGA resin and the pseudo-proline Fmoc-Trp(^{7}Boc)-Thr($^{8}\psi^{Me,Me}$ pro)-OH led to an excellent elongation yield (96%) and to very regular deprotections of N^{α}-Fmocs (Figure 5(B)). This indicates that the aggregation phenomenon related to the difficult sequence was circumvented. Only a slight drop was observed after the introduction of the pseudo-proline. HPLC analysis of the crude PADRE-Muc peptide (Figure 6) revealed a major peak (87%) corresponding to the target peptide. The minor peak (10%) corresponds to a ΔM of +42 Da when compared to the mass of the target peptide and explains the lower purity than the one expected from the elongation yield.

CONCLUSIONS

This study was aimed at determining the optimal conditions under which the 'difficult sequence' of the PADRE epitope could be synthesized in a conventional manner, that is, following the Fmoc/*t*Bu strategy on a standard automated synthesizer. The on-line UV monitoring of the N^{α}-Fmoc removal enabled us to evaluate different intrinsic factors related to the peptide-resin in order to minimize the internal aggregation stabilized by a hydrogen-bonding network. The use of PS resins with low loading, with intermediate loading associated with

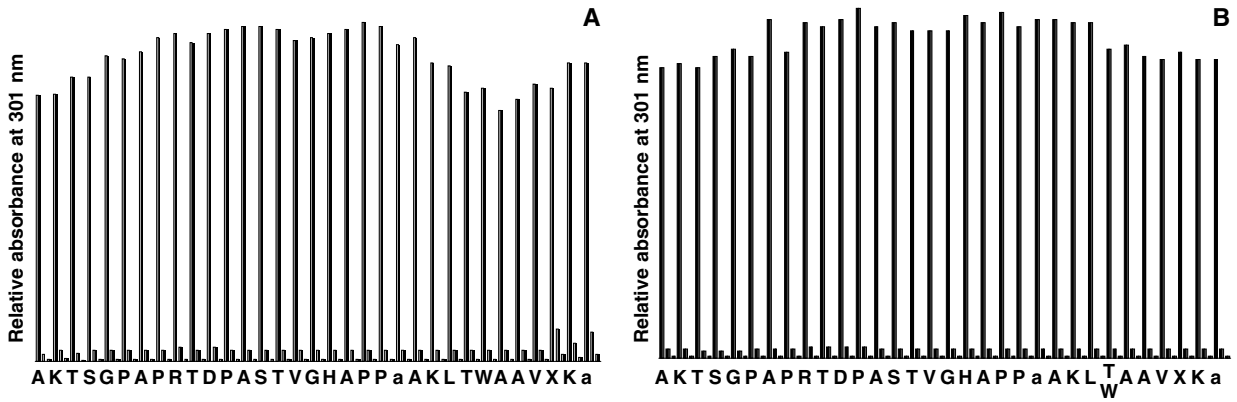

Figure 5 UV N^{α}-Fmoc deprotection trace during the elongation of the PADRE-Muc chimeric peptide (A) on a PEGA resin at a 0.36 mmol/g substitution, elongation yield: 82%; purity of the crude peptide: 83%; (B) on a PEGA resin at a 0.36 mmol/g substitution using a pseudo-proline for the introduction of the Thr-Trp dipeptide, elongation yield: 96%; purity of the crude peptide: 87%.

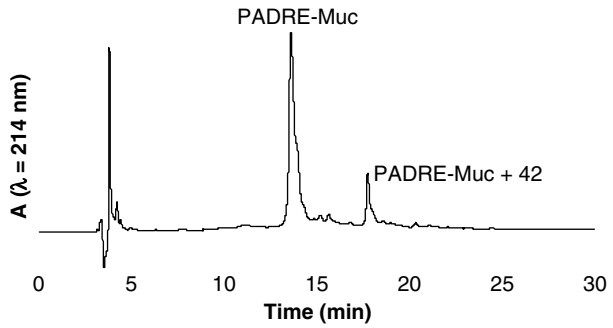

Figure 6 Analytical HPLC profile of the crude PADRE-Muc peptide after synthesis on a PEGA resin substituted at 0.36 mmol/g using a pseudo-proline for the introduction of the Thr-Trp dipeptide.

a pseudo-proline dipeptide, and a PEGA resin with intermediate loading improved the solid-phase synthesis of the PADRE epitope. However, sluggish N^{α}-Fmoc deprotections still occurred. Combining the use of PEGA as a polar resin and a pseudo-proline building block as a protecting group of the amide circumvented this, thus overcoming the 'difficult sequence' problem encountered during the synthesis of PADRE epitope-containing peptides.

MATERIALS AND METHODS

Organic solvents were from SDS (Peypin, France) or Carlo Erba, with DCM, NMP, and piperidine being synthesis grade, and MeCN and MeOH being HPLC grade. Diethyl ether was from SDS (Peypin, France). Water was purified on a Milli-Q reagent system (Millipore). The Fmoc-Rink-amide linker (*p*-{(R,S)-a-[1-(9H-Fluoren-9-nyl)-methoxyformamido]-2,4-dimethoxybenzyl}phenoxyacetic acid) and resins were purchased from Novabiochem (VWR, France), Boc-Ala-4-(oxymethyl)phenylacetic acid from Neosystem (Strasbourg, France). Fmoc-protected amino acids were obtained from Senn Chemicals (Gentilly, France) or Novabiochem (VWR, France). HBTU, purchased from Senn chemicals (Gentilly, France), and HOBt from Fluka were of the highest purity available.

Analytical RP-HPLC was performed using a Merck-Hitachi L7100 pump equipped with a C18 column, nucleosil 300 Å (5 μm, 250 × 4.6 mm), a L-7455 diode array detector and a Merck-Hitachi interface D-7000. Peptides were eluted with a linear gradient of MeCN/H_2O/0.1%TFA. Buffer A was water containing 0.1% TFA and buffer B was MeCN containing 0.1% TFA. The gradient was 20–70% of B over 45 min. The elution was followed at 215 nm. The purity of the crude peptide was estimated by the integration of the HPLC peaks.

Matrix-assisted laser desorbtion ionization (MALDI) analyses were performed on an Autoflex mass spectrometer (Bruker, Wissembourg, France). The calculated masses given correspond to the monoisotopic composition.

General Procedure for Automated Solid-phase Synthesis

Solid-phase peptide synthesis was run on an automated synthesizer 433A from Applied Biosystem using Fmoc/*t*Bu chemistry on a 0.1 mmol scale with HBTU/HOBt as coupling reagents. Ten-equiv excess of protected amino acids, HBTU and HOBt was used and 20-equiv excess of DIEA. The side-chain protecting groups were Lys(Boc), Thr(*t*Bu), Trp(Boc), His(Trt), Ser(*t*Bu), Asp(*t*Bu), Arg(Pbf). The 0.1 mmol scale program (SynthAssist™ version 2) was used with a single coupling followed by capping with acetic anhydride solution. The instrument was coupled to a Perkin Elmer 200S UV/Vis detector. Detector settings were $\lambda = 301$ nm, absorbance range = 0.025 A, rise time = 2 s.

Elongation Yield

The overall elongation yield was calculated as follows: 100 × final loading/original loading. The original loading of each Fmoc-Rink-resin was determined after piperidine treatment (20% in NMP) by UV spectroscopy at 301 nm of the fluorenylmethylpiperidine adduct (ε: 7800 M^{-1} cm^{-1}). Analysis of the fluorenylmethylpiperidine adduct of the last N^{α}-Fmoc removal with 20% piperidine in NMP gave the final loading.

PADRE: H-aKXVAAWTLKAa-NH$_2$ (a: D-Ala; X: cyclohexylalanine). The Fmoc-Rink linker was manually coupled to aminomethyl Tentagel resin (substitution: 0.41 mmol/g) or PEGA resin (substitution: 0.4 mmol/g). The Fmoc-Rink linker (5 equiv) and HBTU (5 equiv) were dissolved in DMF. The solution was then transferred to the aminomethyl resin (1 equiv) placed in a fritted syringe and DIEA (6 equiv) was added after 5 min of stirring. After 4 h, the reactants were removed by filtration and the resin was washed with DMF (3x). The Fmoc-Rink-PS resin was commercially available.

After elongation following the general procedure, an aliquot of the peptide-resin (40 mg) was washed with DCM (3x), and treated with TFA/H_2O/*i*Pr_3SiH, 95 : 2.5 : 2.5. The peptide was then precipitated and washed with ice-cold diethyl ether. The crude peptide was obtained and analyzed by HPLC and mass spectrometry. R_t: 14.10 min; MALDI-MS: 1280.91 (calcd for $C_{62}H_{104}N_{16}O_{13}$ 1280.80).

PADREmuc: H-aKXVAAWTLKAaPPAHGVTSAPDTRPAPGSTAKA-NH-NH$_2$. Boc-Ala-4-(oxymethyl)phenylacetic acid (5 equiv) and HBTU (5 equiv) were dissolved in DMF. The solution was then transferred to an aminomethyl PEGA resin (1 equiv, substitution: 0.4 mmol/g) placed in a fritted syringe and DIEA (6 equiv) was added after 5 min of stirring. After 4 h, the reactants were removed by filtration and the resin was washed with DMF (3x). After elongation following the general procedure, the peptide-resin (200 mg) was treated with TFA/H_2O/*i*Pr_3SiH, 95 : 2.5 : 2.5 for 2 h. The resin was rinsed with CH_2Cl_2 (3x) and NMP (3x) and then treated with 10% DIEA in NMP for 3 × 10 min. After being rinsed with NMP (3x) and H_2O (3x), the peptide-resin was treated with 10% N_2H_4 in H_2O for 2 min. The resin was drained and rinsed with H_2O (3x). The filtrates were pooled, evaporated under vacuum and solubilized in H_2O (86 ml). The crude peptide was analyzed by HPLC and mass spectrometry. R_t: 13.6 min; MALDI-MS: 3362.86 (calcd for $C_{151}H_{247}O_{42}N_{47}$ 3362.86).

Acknowledgements

This work was supported by a grant from 'La ligue contre le cancer, Région Centre'. GAC received a fellowship from the CNRS and La Région Centre.

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