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Influence of polar support for the synthesis of large C-terminal peptide aldehyde: application to chemoselective ligation

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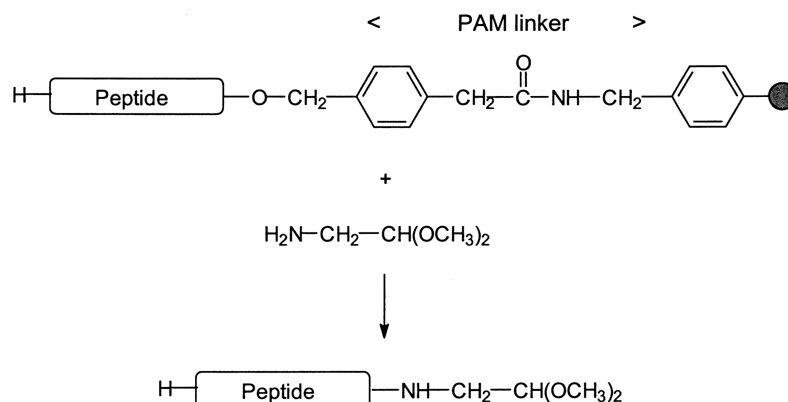
Abstract—Efficient conditions have been developed for the synthesis of large peptide aldehydes from solid support through nucleophilic displacement. Aminolysis of the ester bond between a deprotected peptide and the phenylacetamidomethyl linker with aminoacetaldehyde-dimethylacetal leads to a peptide aldehyde masked as an acetal. Besides the optimization of parameters such as solvents, workup procedure and temperature, the influence of the nature of the polymeric support was crucial. Among the solid supports tested, the poly(ethylene glycol)-poly(acrylamide) resin proved to afford the best cleavage yield. This work underlines that the solid support has to be considered as a co-solvent rather than an inert carrier. Our methodology was further applied to the synthesis of a 33-mer with T-helper activity from the fusion protein of measles virus. The 33-mer peptide aldehyde was then chemoselectively ligated via an oxime bond to an (aminooxy) acetyl peptide with T-cytotoxic activity. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

The use of peptide-based vaccines represents an attractive alternative approach to vaccines composed of rather undefined preparations of whole pathogens.[1] To be efficient, such peptide-based vaccines have to be composed of the three different peptidic epitopes needed to induce a complete immune response, i.e. a B epitope, a T-helper epitope and a T-cytotoxic epitope.[2] This requires the synthesis of large peptides of more than 50 amino acids. The main challenge in peptide synthesis is to establish synthetic routes to homogeneous products of defined covalent structure. This goal could be reached by using recently introduced chemoselective ligation methods which allow for the condensation of unprotected peptide fragments under mild aqueous conditions without any activation step.[3–5] One of the chemoselective methods involves oxime chemistry based on a regiospecific coupling reaction between a peptide aldehyde as the electrophile and an (aminooxy)acetyl peptide as the nucleophile.[5,6] The introduction of the surrogate oxime bond in the backbone of an immunogenic synthetic protein is compatible with the in



Scheme 1.

Keywords: antigens; chemoselectivity; cleavage reaction; peptide aldehyde; polymer effect; solid-phase synthesis.

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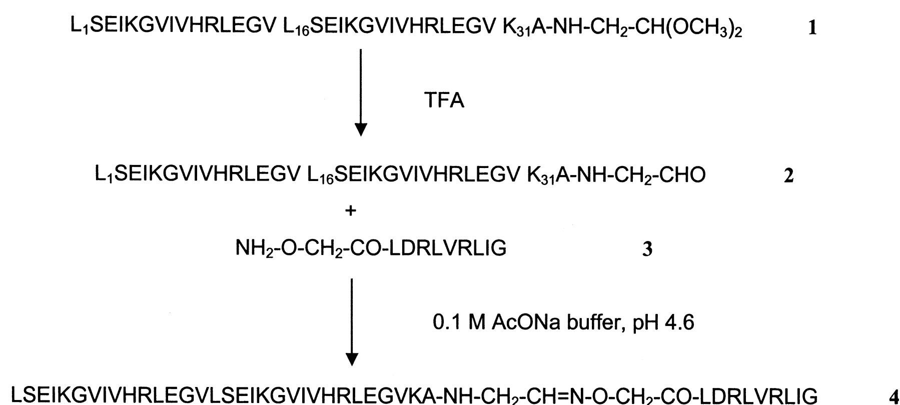


Scheme 2. Chemoselective ligation via oxime bond. LSEIKGVIVHRLEGV: T helper epitope corresponding to the residues 288–302 from the fusion protein of measles virus. LDRLVRLIG: T-cytotoxic epitope corresponding to residues 52–60 from the nucleoprotein of measles virus.

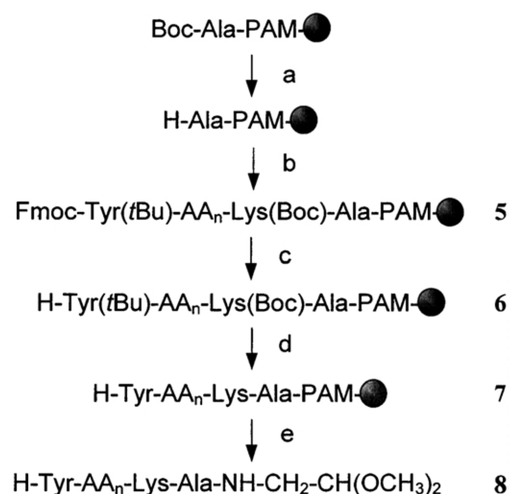
vivo induction of humoral and cellular immune responses.[7,8] In this context, it is highly desirable to develop new methods to build immunogenic peptides composed of varied epitopes. Recently, we have shown that successive unmaskings of aldehyde functions from an acetal and a 2-amino alcohol lead to an α-methyl-aldehyde and an α-oxo-aldehyde, respectively, and affords stepwise chemoselective ligations of two different (aminooxy)acetyl peptides via oxime bonds.[9] This methodology has been achieved on short model peptides. The oxo-aldehyde was classically obtained by periodate oxidation of an N-terminal serine[10] introduced at the ε-NH_2 of a lysine. The C-terminal α-methyl aldehyde protected as an acetal was obtained by aminolysis of the ester bond between the peptide and the phenylacetamidomethyl (PAM) resin with aminoacetaldehyde-dimethylacetal (Scheme 1).[11] Among the methods yielding a C-terminal peptide aldehyde,[12] three have been extended to the synthesis of large peptide aldehydes. The last step of these three methods involved a periodic oxidation either in releasing the peptide aldehyde[13] or in unmasking the aldehyde function.[14,15] In others words, none of these methods are orthogonal to the classical method of Geoghegan, i.e. the periodic oxidation of an N-terminal serine.[10]

We report here the optimization of the aminolysis reaction of the ester bond between the peptide and the PAM resin which leads to a peptide aldehyde masked as an acetal (Scheme 1), the acetal being readily converted to aldehyde by a brief TFA treatment. Among the parameters tested such as solvent, temperature, peptide length, workup procedure, the nature of the polymeric matrix proved to be crucial for the cleavage yield. This methodology was applied further to the synthesis of two peptide acetals, a 21-mer from the Mucine 1 protein and the 33-mer peptide acetal **1** with T-helper activity from the fusion protein of measles virus (Scheme 2). The 33-mer peptide aldehyde **2** was further obtained and chemoselectively ligated via an oxime bond to the T cytotoxic epitope **3** bearing an (aminooxy)acetyl at the N-terminus to yield conjugate **4**.

2. Results and discussion

The target 33-mer peptide aldehyde **2** is composed of a promiscuous T-helper epitope representing the residues 288–302 (LSEIKGVIVHRLEGV) from the fusion protein of measles virus.[16] This T-helper epitope was synthesized in tandem as it has been shown that two copies were more efficient than one copy when coupled to a B-cell epitope or a T-cytotoxic epitope to induce protective antibodies[17] and specific T-cell immune response,[18] respectively. A lysine was introduced at the C-terminus of the two copies to retain the possibility of coupling a serine at its ε-NH_2 for a further transformation into α-oxo-aldehyde by periodic oxidation.[10] Ala_{32} was chosen as the amino acid attached to the PAM resin because bulky amino acids are known to hinder nucleophilic displacement.[19,20] Our own preliminary studies showed that it was also the case with the PAM resin and aminoacetaldehyde-dimethylacetal as the nucleophile.

As a first approach, peptide aldehyde masked as an acetal **8** (Scheme 3) was synthesized on a commercially available Boc-Ala-PAM-polystyrene resin using a Fmoc/*t*Bu solid-phase strategy.[21] We made use of the unique characteristics of the PAM anchor originally developed for the Boc-SPPS



Scheme 3. Stepwise synthesis of C-terminal peptide aldehyde masked as an acetal **8**. (a) TFA 30% in CH_2Cl_2; (b) SPPS elongation using N^{α}-Fmoc amino acid (10 equiv), coupling with DCC, HOBt (10 equiv) in NMP; (c) N^{α}-Fmoc deprotection with piperidine 20% in NMP; (d) side-chain deprotection by TFA/H_2O/phenol/*i*Pr_3SiH 88:5:5:2, followed by neutralization with *i*Pr_2NEt 10% in CH_2Cl_2; (e) aminolysis with $H_2N–CH_2–CH(OCH_3)_2$/CH_2Cl_2/MeOH 2:1:1 at 35°C for 18 h.

Table 1. Peptide grafted on a PAM-PS resin: influence of solvent on the cleavage yield by aminolysis with $H_2N-CH_2-CH(OCH_3)_2$

4-mer YKA-NH-CH_2-$CH(OCH_3)_2$
9-mer YRLEGVKA-NH-CH_2-$CH(OCH_3)_2$
14-mer YGVIVHRLEGVKA-NH-CH_2-$CH(OCH_3)_2$

	Elongation, *x*-mer	Solvent[a]	Cleavage yield (%)[b]	Purity (%)[c]
1	4	CH_2Cl_2/MeOH	80	87
2	9	CH_2Cl_2/MeOH	42	52
3	9	CH_2Cl_2/*i*PrOH	38	100
4	9	DMF	60	94
5	14	DMF	16	89

[a] Aminolysis was performed with $H_2N-CH_2-CH(OCH_3)_2$/solvent(s) 1:1 at 35°C for 18 h. The extraction of the peptide acetal was carried out following procedure A.
[b] Cleavage yield was estimated by UV spectroscopy on the crude *x*-mer peptide acetal ($\varepsilon_{274.8}$ of Tyr=1420 mol^{-1} dm^3 cm^{-1}). Hundred percent was formerly determined by dosage of the fluorenylmethyl-piperidine adduct (ε_{301}=7800 mol^{-1} dm^3 cm^{-1}) after removal of the Fmoc-protecting N^{α}-Tyr with 20% piperidine in NMP.
[c] Estimated by integration of the HPLC peaks at 214 nm.

which is acid- and base-stable.[22,23] After deprotection of N^{α}-Boc with TFA (Scheme 3), the peptide was elongated by Fmoc/*t*Bu chemistry yielding peptidyl resin **5**. Elimination of N^{α}-Fmoc by piperidine followed by a TFA treatment led to the deprotected peptide still attached to the resin **7**. Cleavage of the peptide from the resin was performed by nucleophilic displacement with aminoacetaldehyde-dimethylacetal due to the sensitivity of the PAM anchor to aminolysis,[24,25] and afforded peptide acetal **8**. The fact that the aminolysis was applied on a peptide without any side-chain protection makes this strategy compatible with Asp- and Glu-containing sequences. To evaluate whether the peptide length could have an influence on the cleavage yield, an aliquot of peptidyl resin was put aside during elongation. To quantify the cleavage yield, a Tyr residue was introduced at the N-terminus before cleaving each peptide of determined length.

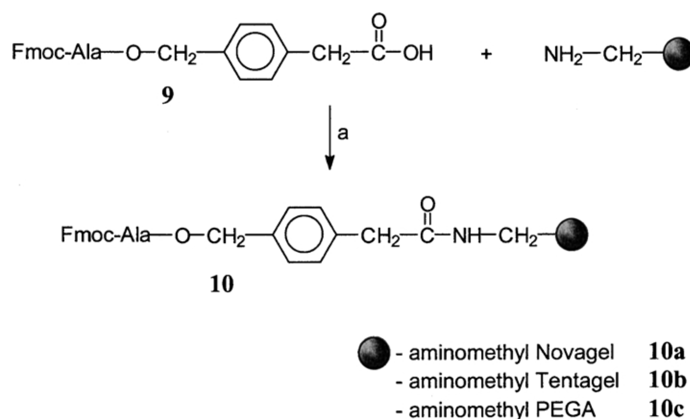
2.1. Optimization of the aminolysis reaction: polystyrene as the solid support

With polystyrene as the solid support, the cleavage reaction was first achieved with $H_2N-CH_2-CH(OCH_3)_2$ in CH_2Cl_2/MeOH at 35°C for 18 h. After workup, the crude mixture was analysed by HPLC and characterized by ESI-MS. The results are shown in Table 1. When the peptide length increased from 4 to 9 residues, the cleavage yield fell dramatically, accompanied by a decrease in purity due to the formation of peptide methyl ester. The sluggish reactivity of the nucleophile made MeOH a good competitor. To diminish this side reaction, co-solvent MeOH was replaced by *i*PrOH (Table 1, entry 3). While the peptide methyl ester completely disappeared, leading to an excellent purity, the cleavage yield did not rise. The use of DMF as a solvent increased the cleavage yield by 20% when compared to the use of CH_2Cl_2 and alcohol (Table 1, entry 4), and the purity of the peptide acetal in the crude mixture was quite good except for an impurity with a Δm of +28 Da. This increase in mass was further located by electrospray tandem mass spectrometry at N^{ε}-Lys. This suggests that the formation of the N^{ε}-formyllysyl adduct was due to the presence of DMF as the solvent.

A considerable drop in cleavage yield by 44% was observed for the 14-mer when compared to the 9-mer, even when using DMF as the solvent (Table 1, entries 4 and 5). This could be ascribed to the impairment of the properties of the polystyrene matrix in terms of swelling and solvation when the peptide became larger.[26] Nevertheless, a few studies[24,25,27] have already been reported on successful cleavage by nucleophilic displacement from peptides linked to PAM polystyrene resin to generate C-terminal-modified peptides from 20 to 33 amino acid in length. In these cases, the side chains of the displaced peptides were protected conferring a poor polarity on the peptide chain not far for being compatible with that of the polystyrene. We reasoned that the use of a more polar matrix for nucleophilic displacement of deprotected peptide still attached to the resin could improve the cleavage yield.

2.2. Advantage of poly(ethylene glycol)-containing resin over polystyrene resin

Poly(ethylene glycol) (PEG) -containing resins are considered as more polar supports than polystyrene (PS) resins.[26] Moreover, the grafted or cross-linked PEG resins present the advantage over PS resin to swell in an expanded range of solvents.[28] Three PEG-containing matrices were used: two



Scheme 4. Synthesis of Fmoc-Ala-PAM resins **10a–c**. (a) TBTU/HOBt/*i*Pr_2NEt.

Table 2. Cleavage yield by aminolysis with $H_2N-CH_2-CH(OCH_3)_2$ of peptide linked to resin based on grafted PEG–PS

14-mer YGVIVHRLEGVKA-NH-CH_2-$CH(OCH_3)_2$
19-mer YLSEIKGVIVHRLEGVKA-NH-CH_2-$CH(OCH_3)_2$
27-mer YIVHRLEGVLSEIKGVIVHRLEGVKA-NH-CH_2-$CH(OCH_3)_2$

	Polymeric matrix	Elongation, *x*-mer	Temperature (°C)	TFA treatment	Workup procedure[a]	Cleavage yield (%)[b]	Purity (%)[c]
1	Novagel	14	35	2 h	A	40	82
2		19	35	2 h	A	15	62
3		19	40	2 h	A	20	ND[d]
4		19	40	2 h	B	34	76
5	Tentagel	19	40	2 h	B	40	35
6		19	40	2 h (2×)	B	41	70
7		27	40	2 h (2×)	B	25	50

Aminolysis was performed with $H_2N-CH_2-CH(OCH_3)_2$/DMF 1:1 for 18 h.
[a] Procedures A and B as described in the experimental part (general procedure for aminolysis).
[b] Cleavage yield was estimated by UV spectroscopy on the crude *x*-mer peptide acetal ($\varepsilon_{274.8}$ of Tyr=1420 mol^{-1} dm^3 cm^{-1}). Hundred percent was formerly determined by dosage of the fluorenylmethyl-piperidine adduct (ε_{301}=7800 mol^{-1} dm^3 cm^{-1}) after removal of the Fmoc-protecting N^{α}-Tyr by 20% piperidine in NMP.
[c] Estimated by integration of the HPLC peaks at 214 nm.
[d] Not determined.

grafted PEG–PS to test the environmental and the spacer effect, Novagel resin[29] with functionality attached to the polystyrene core and PEG spacers used only as modifiers, Tentagel resin[30] with functionality attached to the end of the linear PEG spacer, and one matrix based on cross-linked PEG, the poly(ethylene glycol)-poly(acrylamide) (PEGA) resin which does not possess a polystyrene core.[31]

To test the different matrices, we had to introduce acid **9** (Scheme 4) on the three different aminomethyl resins, i.e. Novagel, Tentagel, PEGA, as the corresponding Boc or Fmoc amino acid PAM resins are not commercially available. Acid **9** was synthesized as already described,[23,32] and was attached to aminomethyl resin using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) activation[33] leading to the Fmoc-Ala-PAM resins **10a–c**.

2.3. Grafted PEG–PS resins

The increase in cleavage yield was 24% for the 14-mer peptide acetal when using Novagel resin (Table 2, entry 1) as compared to the use of PS resin (Table 1, entry 5). This is in good agreement with our hypothesis about the importance of compatible polarity for both the deprotected peptide chain and the polymeric matrix and with what has been pointed out by Barany[34,35] on the crucial role of grafted-PEG in terms of environmental effect. However, the yield still decreased when the peptide chain was lengthened (Table 2, entries 1 and 2). At this stage, we decided to check other parameters such as temperature and workup procedure. Increase in temperature does not play a crucial role in improving the cleavage yield (Table 2, entries 2 and 3). In contrast, alterations in the workup (Table 2, entries 3 and 4), especially by enhancing washings of the resin with 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) and TFE (workup procedure B), lead to an increase in cleavage yield by 14%, underlining the importance of the extraction procedure to remove the peptide from the matrix network. Under similar conditions and with Tentagel as polymeric matrix, the purity of peptide acetal was surprisingly poor (Table 2, entry 5). ESI-MS characterization revealed an incomplete deprotection of Ser(*t*Bu) and Arg(Pbf). To assure a complete removal of the side-chain protecting groups, the TFA procedure was extended by adding a second 2-h treatment. This afforded an acceptable purity without altering the cleavage yield (Table 2, entry 6). About the location of the grafted-PEG on the polystyrene core, no great differences in cleavage yield were found (Table 2, entries 4 and 6). Despite the use of PEG-grafted matrices, the cleavage yield became low when elongating the peptide sequence (Table 2, entries 6 and 7), and probably unacceptable for any longer peptide acetal.

2.4. PEGA resin as a cross-linked PEG resin

We assumed that a polymeric matrix without a polystyrene core could improved the cleavage yield. The aminomethyl

Table 3. Cleavage yield by aminolysis with $H_2N-CH_2-CH(OCH_3)_2$ of peptide linked on a PAM-PEGA resin

14-mer YGVIVHRLEGVKA-NH-CH_2-$CH(OCH_3)_2$
19-mer YLSEIKGVIVHRLEGVKA-NH-CH_2-$CH(OCH_3)_2$
27-mer YIVHRLEGVLSEIKGVIVHRLEGVKA-NH-CH_2-$CH(OCH_3)_2$

	Polymeric matrix	Elongation, *x*-mer	Cleavage yield (%)[a]	Purity (%)[b]
1	PEGA	14	91	83
2		19	91	68
3		27	90	58

Aminolysis was performed with $H_2N-CH_2-CH(OCH_3)_2$/DMF 1:1 at 40°C for 18 h. The extraction of the peptide acetal was carried out following procedure B.
[a] Cleavage yield was estimated by UV spectroscopy on the crude peptide acetal ($\varepsilon_{274.8}$ of Tyr=1420 mol^{-1} dm^3 cm^{-1}). Hundred percent was formerly determined by dosage of the fluorenylmethyl-piperidine adduct (ε_{301}=7800 mol^{-1} dm^3 cm^{-1}) after removal of the Fmoc-protecting N^{α}-Tyr by 20% piperidine in NMP.
[b] Estimated by integration of the HPLC peaks at 214 nm.

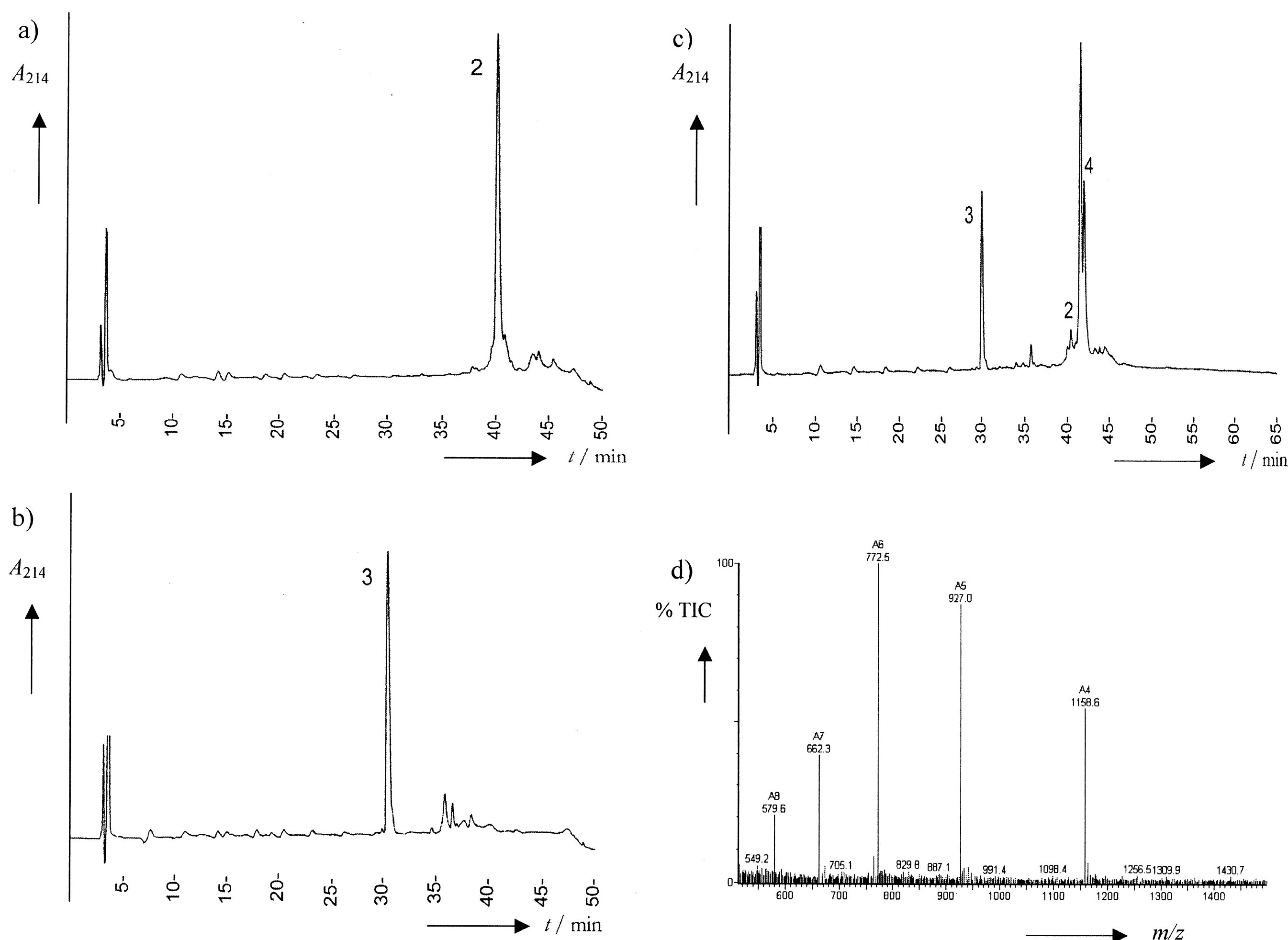


Figure 1. Chemical ligation between the 33-mer peptide aldehyde **2** and the (aminooxy)acetyl peptide **3**. Analytical HPLC (C8) of: (a) the peptide aldehyde **2**; (b) the (aminooxy)acetyl peptide **3** with the following gradient: 5% AcN in H_2O for 2 min, 5–50% AcN in H_2O containing 0.1% TFA over 40 min at a flow rate of 1 ml/min. (c) Analytical HPLC (C8) of the ligation mixture between peptide **2** and **3** with the following gradient: 5% AcN in H_2O for 2 min, 5–70% AcN in H_2O containing 0.1% TFA over 60 min at a flow rate of 1 ml/min. (d) Electrospray ionization mass spectrum of the purified peptide conjugate **4**. The $4H^+$ (A4) to $8H^+$ (A8) charge states of the peptide correspond to a mass of 4629.21±1.07 Da; calculated average isotope composition 4629.56 Da.

PEGA resin[31] which contains only a cross-linked PEG was then tested. Cleavage yields obtained after aminolysis with aminoacetaldehyde-dimethylacetal were excellent and stable whatever the peptide length (Table 3). The purity was very good for the 14-mer (Table 3, entry 1). No problem was encountered in terms of incomplete deprotection of Ser and Arg side chains. The lower purity revealed for the 19- and 27-mer (Table 3, entries 2 and 3) was due to the presence of three peaks corresponding to side-products, two with a Δm of +28 Da and one with a Δm of +56 Da when compared to the target peptide acetal. They were attributed to various formylation of the two Lys residues as described above.

The absence of polystyrene core could confer to the PEGA resin a suitable polarity allowing the proper solvation of both the peptide and the resin. Moreover, as the longest peptide acetals are not soluble in DMF in solution, the attachment of the peptide to PEGA resin must contribute to their solvation. For the synthetic transformation described here, PEGA resin did not behave as an inert carrier as Burgess pointed out concerning the role of the support for S(n)r and S(n)2 macrocyclization on resin,[39] but rather as a co-solvent. Czarnik[40] has summarized some key observations on this subject and emphasized that polymeric matrices are like solvents. Nevertheless, we cannot rule out the swelling properties of PEGA resin which do not change when elongating the peptide.[36] They could play a role by favouring the good diffusion of macromolecules into PEGA resin as already proposed for enzymatic reactions[37] and chemoselective ligation.[38]

To strengthen the interest of PEGA resin for the synthesis of peptide acetal, we decided to synthesize an other sequence which has no concern with the T-helper epitope from measles virus. We chose a 21-mer peptide [Ac-PPAHGVT-SAPDTRPAPGSTA-NH–CH_2–$CH(OCH_3)_2$] from the Mucine 1 protein.[41] Starting from the Fmoc-Ala-PAM-PEGA-resin **10c**, the Muc1-acetal was obtained in pure form after HPLC purification with an overall yield of 51%.

2.5. Chemoselective ligation

The very encouraging results obtained with the PEGA resin prompted us to undertake the synthesis of the 33-mer

peptide acetal **1** (Scheme 2) containing two copies of T-helper epitope on PEGA resin. Starting from the Fmoc-Ala-PAM-PEGA resin **10c**, the 33-mer peptide acetal **1** was obtained with a 18% overall yield. This yield results from the poor yield of HPLC purification (24%). Apart from the presence of formylated side products, some target peptide must have been lost on semi-preparative column due to the amphipathic character of the T-helper epitope moiety.[16,42] To the best of our knowledge, this peptide represents one of the longest peptide aldehydes synthesized from solid support.[13,15] Before ligation, the unmasking of the aldehyde group was performed by a brief TFA treatment to give peptide aldehyde **2**, and the peptide solution was buffered at pH 4.6 with 0.1 M AcONa. The T-cytotoxic epitope **3** corresponding to the residues 52–60 from the nucleoprotein of measles virus was synthesized by Fmoc/*t*Bu chemistry on a commercially available Fmoc-Gly-Wang-resin. Following elongation, aminooxy acetic acid (Aoa) was introduced at the N-terminus as Boc-Aoa.[11,43] TFA treatment released (aminooxy)acetyl peptide **3** which was HPLC-purified with careful handling to minimize side reactions on the aminooxy group.[44,45] The chemoselective ligation between the peptide aldehyde **2** and the (aminooxy)acetyl peptide **3** in 1.2-fold excess was conducted at pH 4.6 in 0.1 M AcONa. It was near-quantitative over 150 min (Fig. 1). The conjugate **4** was chromatographied as a double peak which is likely due to the *syn*- and *anti*-forms of the oxime bond.[46] It was recovered by HPLC purification with a yield of 41% and characterized by ESI-MS (Fig. 1(d)). Finally, the target peptide **4** was obtained in pure form with an overall yield of 10% starting from the resin substitution of the limiting compound, i.e. the peptide aldehyde **2**.

2.6. Conclusion

The potential usefulness of the aminolysis of the ester bond between a peptide and the PAM resin with aminoacetaldehyde-dimethylacetal was demonstrated for the synthesis of large peptide acetal. Moreover, this work underscores the importance of the polymeric matrix when studying supported reaction and provides new insights into nucleophilic cleavage releasing C-terminus-modified peptides. To favour nucleophilic displacement of unprotected peptide, the key parameter is the nature of the polymeric support whose polarity has to be compatible with the peptide chain. Finally, the successful synthesis of large peptide aldehyde masked as an acetal permits a subsequent chemoselective ligation. This work opens the way to the synthesis of complex linear or multi-branched peptides where peptides bearing one or more aldehyde functions will serve as precursors for successive ligations.

3. Experimental

3.1. General

Organic solvents were from SDS (Peypin, France), with DCM, NMP, and piperidine being synthesis grade, AcN and MeOH being HPLC grade. Diethyl ether was from SDS (Peypin, France) and the higher purity from Sigma (St Quentin Fallavier, France). DMF from Carlo Erba (Val de Reuil, France) was kept stored on 4 Å sieves after having been distilled on Z-Gly-ONp. TFA was from SDS (Peypin, France). Water was purified on a Milli-Q reagent system (Millipore). Boc-Ala-PAM-copoly(styrene 1% divinylbenzene) resin was from Neosystem (Strasbourg, France). Fmoc-Ala-Wang-resin, Novagel, Tentagel (NovaSyn TG) and PEGA resins were purchased from Novabiochem (Meudon, France). Fmoc-protected amino acids were obtained from Senn Chemicals (Gentilly, France) or Novabiochem (Meudon, France). Aminoacetaldehyde-dimethylacetal and aminooxy acetic acid (Aoa) were from Sigma (St. Quentin Fallavier, France). N^{α}-Boc-protected Aoa was obtained according to Offord.[43] Coupling reagents were purchased from commercial sources and were of the highest purity available.

Analytical and semi-preparative RP-HPLC were performed using a Merck-Hitachi L6200A pump equipped with a C18 column, Lichrospher 100 Merck (5 μm, 250×4 mm^2), Lichrosorb 100 Merck (7 μm, 250×10.5 mm^2), or a C8 column Vydac 300 Å (5 μm, 250×4 mm^2), Vydac 300 Å (5 μm, 250×10.5 mm^2), a 655A variable wavelength UV monitor, and a Merck-Hitachi integrator D-7500. Peptides were eluted with a linear gradient of AcN/H_2O/0.1% TFA. Buffer A was water containing 0.1% TFA, buffer B was AcN containing 0.1% TFA, buffer C was H_2O/AcN, 40:60 containing 0.1% TFA. Gradient A: 5–50% C in A over 40 min. Gradient B: 20–70% C in A over 40 min. Gradient C: 30–100% C in A over 40 min. Gradient D: 5% B in A for 5 min, 5–100% B in A over 45 min. Gradient E: 5% B in A for 2 min, 5–50% B in A over 40 min. Gradient F: 5% B in A for 2 min, 5–70% B in A over 60 min. Gradient G: 8–60% C in A over 40 min. The elution was followed at 214 nm.

Electrospray mass spectrometry (ESI-MS) analyses were performed on a triple quadrupole mass spectrometer (Quattro II, Micromass, Manchester, UK). The calculated masses given correspond to the average isotope composition. Depending on the voltage applied to the sample cone, the peptide acetals lose one or two CH_3OH. For more details concerning this phenomenon, see our recent study.[47] We report here only the data corresponding to the unfragmented peptides. ^1H NMR spectra were recorded on a Brücker 300 MHz spectrometer.

3.2. General procedure for solid-phase synthesis

Solid-phase peptide synthesis was run on an automated synthesizer 431A from Applied Biosystem using Fmoc/*t*Bu chemistry at 0.1 mmol scale with DCC/HOBt as coupling reagents. The small-scale programme purchased from the manufacturer was followed with polystyrene, Novagel and Tentagel resins, whereas the standard scale programme was used with the PEGA resin to properly swell the resin. Tenfold excess was used for protected amino acids and coupling reagents. The side-chain protecting groups used were Tyr(*t*Bu), Lys(Boc), Asp(*t*Bu), Glu(*t*Bu), Ser(*t*Bu), Thr(*t*Bu), His(Trt), and Arg(Pbf). Fmoc group was removed with 20% piperidine in NMP for 3 min (3×).

3.3. General procedure for aminolysis

Small-scale test cleavages were performed using 20–50 mg

of peptide-resin in a 10 ml home-made reactor equipped with a double-walled jacket for thermostating and a refrigerant. Large-scale cleavage of peptide resin was performed using a 40 ml home-made double-walled reactor. Before each aminolysis, α-NH_2 and side chains were deprotected. N^{α}-Fmoc was manually removed by 20% piperidine in DMF (3×) followed by washing the resin with CH_2Cl_2 (4×). Side chains were deprotected with TFA/phenol/H_2O/Triisopropylsilane, 88:5:5:2 for 2 h. The resin was washed with CH_2Cl_2 (4×) and neutralized with $i$$Pr_2NEt$/$CH_2Cl_2$ 1:9 followed by washings with DMF (3×). The absorbance of the fluorenylmethyl-piperidine adduct was measured at 301 nm (ε=7800 mol^{-1} dm^3 cm^{-1}) to quantify the peptide to be cleaved. N^{α}-Fmoc deprotection, TFA treatment and aminolysis were carried out in the same reactor to minimize losses of peptidyl resin by handling. Aminolysis with aminoacetaldehyde-dimethylacetal was conducted in the presence of given solvents for 18 h at a determined temperature under gentle magnetic stirring. After cleavage, two procedures were followed to recover peptide acetal. Procedure A: the resin was drained and washed with CH_2Cl_2, MeOH, H_2O 0.1% TFA, HFIP, CH_2Cl_2, MeOH, CH_2Cl_2. Filtrates were polled and evaporated under vacuum. The resulting oil was dissolved in a given volume of distilled water. pH was adjusted at 7 with TFA. The cleaved peptide was quantified by measuring the absorbance of tyrosine at 274 nm (ε= 1420 mol^{-1} dm^3 cm^{-1}). Procedure B (for the longest peptide acetals which are not soluble in DMF): aminolysis mixture solution containing DMF and aminoacetaldehyde-dimethylacetal was filtered off and peptide extraction from the resin was then performed by successive washings with CH_2Cl_2 (2×), TFE (3×), HFIP (3×). After evaporation under vacuum, the oil was dissolved in a given volume of distilled water. pH was adjusted at 7 with TFA. The absorbance of Tyr was measured at 274 nm (ε=1420 mol^{-1} dm^3 cm^{-1}).

3.4. Fmoc-Ala-PAM-resins 10a–c

The acid **9** was synthesized according to Wong and co-workers.[23] Acid **9** (262 mg, 0.57 mmol) was preactivated in DMF (2 ml) with TBTU (167 mg, 0.57 mmol), HOBT-H_2O (88 mg, 0.57 mmol) and $i$$Pr_2NEt$ (196 μl, 0.57 mmol) for 45 min. The solution was then transferred on aminomethyl Novagel resin (500 mg, 0.76 mmol/g) in DMF (3 ml). After 18 h, the reactants were removed by filtration. A second coupling was performed under the same conditions. The resin was washed with DMF (2×) before capping with DMF/Ac_2O/$i$$Pr_2NEt$, 2:2:1 (2×10 min). Yield and final substitution were determined by UV spectroscopy of the fluorenylmethyl-piperidine adduct at 301 nm (ε=7800 mol^{-1} dm^3 cm^{-1}) after removing N^{α}-Fmoc by 20% piperidine in DMF (4 ml, 3×3 min) followed by CH_2Cl_2 washings (3×). A similar procedure was followed with aminomethyl Tentagel and PEGA resin. **10a**: yield 81%, final substitution 0.46 mmol/g; **10b**: yield 75%, final substitution 0.32 mmol/g; **10c**: yield 91%, final substitution 0.36 mmol/g.

3.5. Synthesis of 4-, 9-, 14-, 19-, 25-mer peptide acetals: optimization of the aminolysis reaction

Peptide elongation was performed on a N^{α}-protected Ala-PAM resin as specified in Tables 1–3. The general procedure was followed for the elongation. Side chains were deprotected with TFA/phenol/H_2O/Triisopropylsilane, 88:5:5:2 for 2 h, except where especially notified (Table 2). Solvents for aminolysis and workup procedure were specified in each table. The crude aminolysis mixture was analysed by C18 RP-HPLC. 4-mer: R_t (gradient A): 16.67 min, ESI-MS: $[M+H]^+$ 468.39 (calcd 468.57); 9-mer: R_t (gradient A): 29.20 min, ESI-MS: M, 1021.79±0.27 (calcd 1022.19); 14-mer: R_t (gradient A): 40.28 min, ESI-MS: M, 1527.38±0.23 (calcd 1527.81); 19-mer: R_t (gradient B): 29.23 min, ESI-MS: M, 2098.67±0.84 (calcd 2098.49); 25-mer: R_t (gradient C): 28.31 min, ESI-MS: M, 3001.21±0.66 (calcd 3002.57).

3.6. Muc1-acetal [Ac-PPAHGVTSAPDTRPAPGSTA-NH–CH_2–$CH(OCH_3)_2$]

Fmoc-Ala-PAM-PEGA **10c** (195 mg) was introduced into a reactor for solid-phase synthesis. The elongation was conducted as described in the general procedure with the standard scale programme. After completion of the elongation and a final acetylation, 125 mg of the peptidyl resin were introduced into a double-walled reactor. N^{α}-Fmoc was deprotected with 20% piperidine in DMF (4 ml) followed by washings with CH_2Cl_2 (3×4 ml). Side chains were deprotected with TFA/phenol/H_2O/triisopropylsilane, 88:5:5:2 for 2 h. The resin was washed with CH_2Cl_2 (4×), neutralized with $i$$Pr_2NEt$/$CH_2Cl_2$ 1:9 (4 ml) and then washed with DMF (3×). Aminoacetaldehyde-dimethylacetal (2 ml) and DMF (2 ml) were added to the peptidyl resin and left gently stirring for 18 h at 40°C. Workup was carried out according to procedure B and the crude peptide acetal was submitted to C18-RP-HPLC. After lyophilization, 16 mg were obtained as a white powder corresponding to an overall yield of 51% according to resin **10c** substitution. R_t (gradient G) 26.9 min; ESI-MS: 2015.8±0.12 Da, calcd 2016.13.

3.7. 33-mer Peptide acetal 1

Fmoc-Ala-PAM-PEGA **10c** (278 mg) was introduced into a reactor for solid-phase synthesis. The elongation was conducted as described in the general procedure with the standard scale programme. After completion of the elongation, 295 mg of the peptidyl resin were introduced into a double-walled reactor. N^{α}-Fmoc was deprotected with 20% piperidine in DMF (4 ml) followed by washings with CH_2Cl_2 (3×4 ml). Side chains were deprotected with TFA/phenol/H_2O/triisopropylsilane, 88:5:5:2 for 2 h. The resin was washed with CH_2Cl_2 (4×), neutralized with $i$$Pr_2NEt$/$CH_2Cl_2$ 1:9 (4 ml) and then washed with DMF (3×). Aminoacetaldehyde-dimethylacetal (2 ml) and DMF (2 ml) were added to the peptidyl resin and left gently stirring for 18 h at 40°C. Workup was carried out according to procedure B and the crude peptide acetal was lyophilized. Seventy-one milligrams of this material were submitted to C18-RP-HPLC. After lyophilization, 17 mg were obtained as a white powder (recoverable yield: 24%) corresponding to an overall yield of 18% according to resin **10c** substitution. R_t (gradient D) 28.9 min; ESI-MS: 3565.58±0.5 Da, calcd 3566.22.

3.8. (Aminooxy)acetyl peptide 3

Peptide **3** was prepared using Fmoc-Gly-Wang-resin (204 mg, 0.1 mequiv). Elongation was performed according to the general procedure. Boc-Aoa was introduced as a usual amino acid but after being dissolved in DMSO. After completion of the elongation, the peptide was deprotected and cleaved from the resin with TFA/phenol/H_2O/$i$$Pr_3SiH$, 88:5:5:2 and then precipitated and washed with ice-cold diethyl ether without carbonyl-containing compound. The crude peptide was taken up by water, lyophilized and purified by C18 RP-HPLC. Peptide **3** was recovered as a white powder after lyophilization with an overall yield of 31%. R_t: 30.5 min (gradient E); ESI-MS: 1126.8±1.00 Da, calcd 1127 Da.

3.9. Peptide conjugate 4

Peptide acetal **1** (7.5 mg, 2.1 μmol) was treated with TFA/H_2O, 90:10 (2 ml) for 10 min. After evaporation of the TFA under vacuum, peptide aldehyde **2** (R_t (gradient E): 39.9 min, ESI-MS: M 3519.02±0.71 Da, calcd 3520.22; M+H_2O 3537.43± 0.94 Da, calcd 3538.22) was dissolved in 0.1 M NaOAc, pH 4.6 (200 μl). To this solution was added (aminooxy)-acetyl peptide **3** (2.84 mg, 2.5 μmol) dissolved in 0.1 M NaOAc, pH 4.6 (1.6 ml), and the reaction mixture was left stirring for 150 min. Peptide-conjugate **4** was purified by C8-RP HPLC as a double peak; R_t: 41.52 min (gradient F), ESI-MS: 4629.06±1.06 Da; R_t: 41.96 min, ESI-MS: 4629.21±1.07 Da, calcd 4629.56 Da.

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