Synthesis of Branched Oxime-Linked Peptide Mimetics of the MUC1 Containing a Universal T-Helper Epitope

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Abstract: Our goal was to develop mimics of MUC1, highly immunogenic to induce an efficient immune response against the tumor-associated form of MUC1, and sufficiently different from the natural antigen to bypass the tolerance barrier in humans. With the aim of obtaining a well-defined peptide construct as a means of evoking the precise immune responses required in immunotherapy, we synthesized artificial mimics of the MUC1 protein composed of two MUC1 repeat units of inverse orientation and a universal T-helper epitope. To synthesize these heteromeric peptide constructs, we followed a convergent approach using chemoselective ligation based on oxime chemistry. A stem peptide was first synthesized bearing two orthogonally masked aldehydes. After successive deprotection, two oxime bonds can be specifically generated. The proposed strategy proved to be concise and robust, and allowed the synthesis of the tri-branched protein in a very satisfactory yield. The different constructs were tested for their ability to generate antibodies able to recognize the MUC1 protein.

Keywords: antibodies · chemoselective ligation · oxime · peptides

Introduction

In recent years, the cell surface mucin MUC1 has generated great interest as a potential target for the immunotherapy of human tumors of epithelial origin.[1] The MUC1 protein is a highly glycosylated protein whose peptide backbone mainly consists of a 20-amino acid sequence repeated in tandem 30 to 90 times.[2] A number of adenocarcinomas abundantly express on their cell surface and secrete an underglycosylated form of the MUC1 tandem repeat sequence. Underglycosylation exposes on cancer cells the immunodominant APDTRPA peptide sequence that is cryptic on normal cells as well as tumor-associated saccharide antigens such as Tn-(GalNAcαSer/Thr), sialyl-Tn-(NeuAcα2,3GalNAcαSer/Thr) and T-(Galβ-1,3GalNAcαSer/Thr) antigens.[3] These cancer specific modifications make the MUC1 protein a good target for immune intervention.[4] Therapeutic vaccination attempts were carried out with the MUC1 protein, as either the underglycosylated protein isolated from tumoral tissues, the recombinant proteins or synthetic peptides of different lengths.[5] It has been reported that a linear peptide chain has to be composed of at least four repeat units in order to be immunogenic.[6] However, MUC1 peptides corresponding to only one repeat of the 20-amino acid sequence linked to KLH were able to induce an effective immune response in women suffering from breast cancer.[7] These results strongly suggest that an artificial presentation of the MUC1 repeat peptide is able to improve the immune response. Nevertheless, the use of KLH, a very immunogenic protein, generates undesirable immune responses, such as carrier-induced epitope suppression and irrelevant antibody production.[8] In addition, the structure of the immunogen is completely undefined. As an alternative, it is possible to couple a peptide or a glycopeptide to a universal T-helper epitope,[9] that is, a T-helper epitope able to bind to many human MHC class II proteins in order to be efficient in a large population.[10]

Our long-term goal has been to develop glycoprotein mimics of MUC1, highly immunogenic to induce an efficient immune response against the tumor-associated form of the

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Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author. Supporting information: Figures S1 and S2: Analytical chromatograms of crude Aoa-Muc **3** and Aoa-PADRE **4**. Figure S3: Analytical HPLC profile of the ligation reaction leading to **6** after 18 h. Figure S4: Analytical HPLC profile of Muc(Muc)acetal **6** after 6 min-TFA treatment. Figure S5: Analytical HPLC profile of Muc(Muc)acetal **6** after lyophilization in the presence of Aoa-PADRE **2**. Figure S6: Analytical HPLC profile of Muc(Muc)Aoa **7**.

 DOI: 10.1002/chem.200400780

MUC1, and sufficiently different from the natural antigen to bypass the tolerance barrier in humans. With the aim of obtaining a well-defined peptide construct as a means of evoking the precise immune responses required in immunotherapy, we decided to develop, at first, the synthesis of unglycosylated artificial mimics of the MUC1 protein composed of two MUC1 repeat units and a universal T-helper epitope. To be different from tumor-related antigens, the two repeat units and the T-helper epitope are presented on a tri-branched construct with the two MUC sequences being inversely oriented, that is, one with a free N-terminus, the other with a free C-terminus (Figure 1). In addition, the branches are linked through pseudopeptide bonds to each other and to the T-helper epitope to render the artificial tumor antigen slightly different from the endogenous antigen with the aim of bypassing the immunological tolerance. For the T-helper epitope, we chose PADRE (Pan DR Epitope), an artificial peptide sequence engineered to be a highly efficient universal T-helper epitope.[11] In addition to being recognized by many human DR alleles, it also binds to several murine alleles including those from C57Bl/6 mice which were used to test the immune response in vivo.

To synthesize these small proteins, we followed a convergent approach using chemoselective ligation based on oxime chemistry, that is, the condensation of an unprotected peptide aldehyde to an unprotected aminooxypeptide.[12] Oxime bonds were chosen for chemical and biological reasons. The high efficiency and selectivity of oximation reactions have been demonstrated successfully[13] as well as its stability in a wide range around the physiological pH.[14] Moreover, the introduction of the surrogate oxime bond in the peptide backbone of an immunogenic synthetic protein is compatible with the in vivo induction of humoral and cellular immune responses.[15]

It is possible to successively generate two oxime bonds on a stem peptide by orthogonally protecting either the amine of the aminooxy partner[16] or the aldehyde partner.[17] As the latter presents the interesting feature of installing a reactive aldehyde group at the C-terminus, we decided to explore this strategy. The critical point of our approach, besides the synthesis of the stem peptide bearing two masked aldehydes (Figure 1), was the order of unmasking the aldehyde functions for two successful chemoselective ligations. The proposed strategy proved to be concise and robust, and allowed the synthesis of the tri-branched protein in a very satisfactory yield. The different constructs were tested for their ability to induce the generation of antibodies which recognize the MUC1 protein.

Results and Discussion

To afford the tri-branched heteromeric construct **1**, three starting building blocks were needed (Figure 1): two peptides bearing an (aminooxy)acetyl (Aoa) group, that is, Aoa-Muc **3** and Aoa-PADRE **2** and the Muc peptide bearing two masked aldehydes **4**, a 2-amino alcohol (a serine installed at the ε-NH_2 of an additional C-terminal lysine) and an acetal (a glycinal masked as an acetal at the C-terminus). Our synthetic strategy was based on two key features: i) the synthesis of the stem peptide bearing two masked aldehydes, one of which is located at the C-terminus; ii) the successive formation of two oxime bonds.

The elongation of Aoa-PADRE **2** and Aoa-Muc **3** was carried out by a Fmoc/*t*Bu strategy starting from a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink-amide resin)[18] and a *p*-benzyloxybenzyl alcohol resin (Wang resin),[19] respectively. The purity was estimated by HPLC integration to 82% for **2** and 80% for **3**. Taking into account the excellent purity of the crude peptide and the high reactivity of the Aoa group with carbonyl-containing compounds even present as traces in solvents,[13a,20] peptides **2** and **3** were engaged in an oximation reaction without HPLC purification.

For the synthesis of **4**, two masked aldehydes were installed on a stem peptide. Besides the classical generation of an aldehyde by 2-amino alcohol oxidation,[21] the second aldehyde was introduced at the C-terminus by nucleophilic displacement of the ester bond between the peptide and the PAM linker with aminoacetaldehyde-dimethylacetal.[22] The PEGA resin was used as a polymeric matrix to facilitate the aminolysis of a peptide longer than ten amino acids.[22b] The

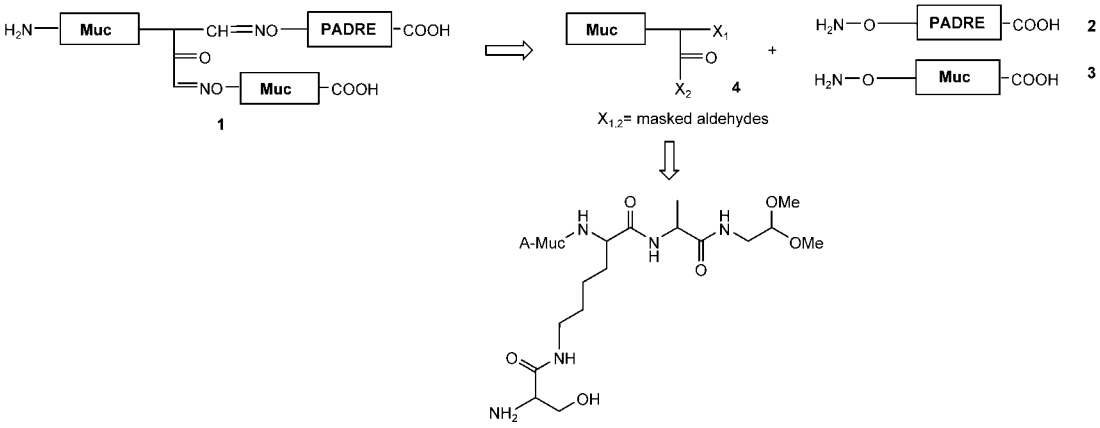
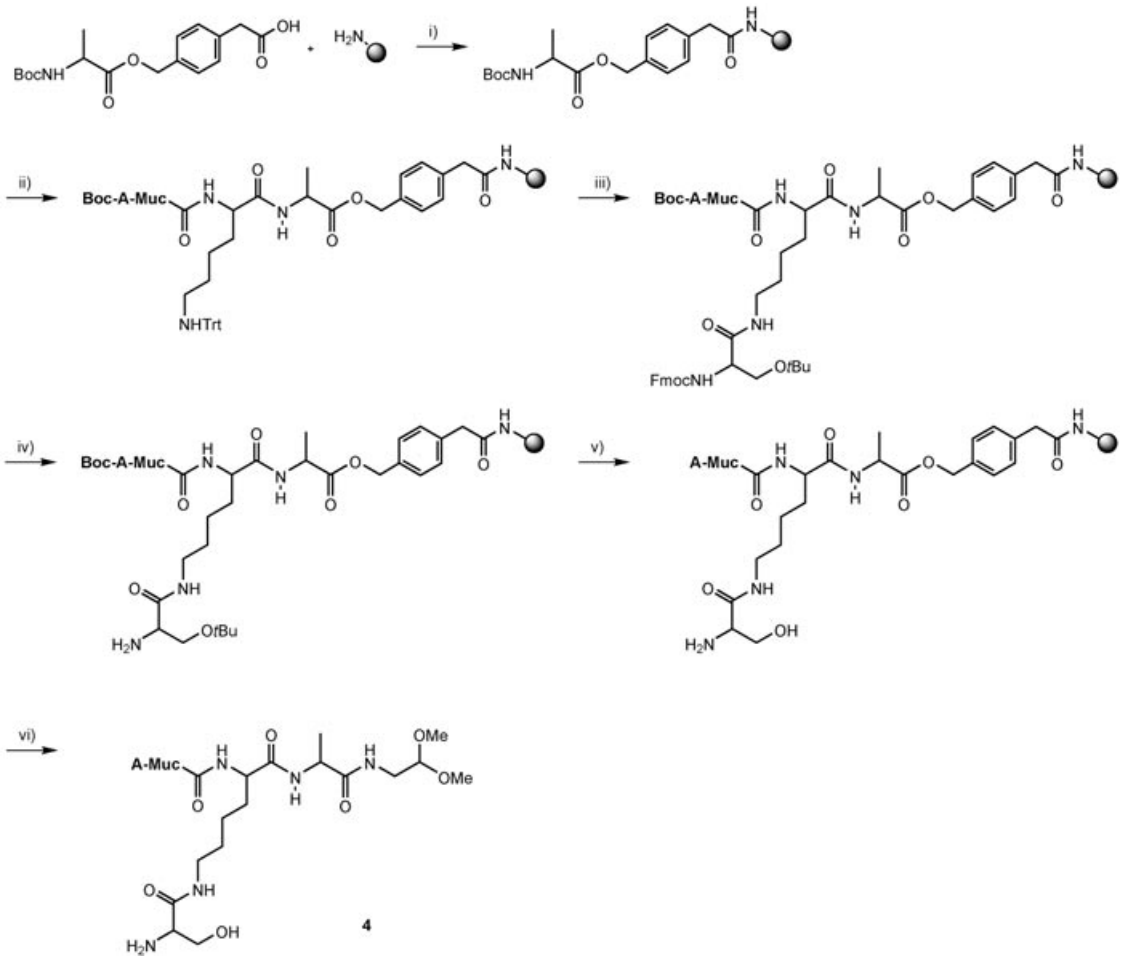


Figure 1. Synthesis of the tri-branched peptide Muc(Muc)Padre **1** (Muc: PPAHGVTSAPDTRPAPGSTA; an additional alanyl residue (A) was added at the N-terminus of the stem peptide dialdehyde **4**; PADRE: aKXVAAWTLKa with a being the D-Ala and X the cyclohexylalanyl residue).

synthesis of the stem peptide **4** with two masked aldehydes was proceeded as shown in Scheme 1. The PAM linker was attached on the PEGA resin by using, this time, the commercially available Boc-Ala-4-(oxymethyl)phenylacetic acid linker. The completion of the reaction was checked by Kaiser's test.[23] The use of the in situ protocol described for Fmoc-chemistry[24] was needed to afford a quantitative coupling whereas the classical preactivation protocol afforded an incomplete reaction even after a double coupling. After Boc removal by TFA treatment and DIEA neutralization, the elongation was carried out following the Fmoc/*t*Bu strategy. To quantitatively install the seryl residue at the N^{ε}-Lys, several protecting groups were tested to be orthogonal to the Fmoc/*t*Bu strategy and the PAM linker. In a first attempt, the seryl residue was introduced before the end of the elongation. Taking advantage of the PAM linker's property of being stable to TFA and piperidine treatment,[25] the lysyl residue was introduced as the inexpensive Fmoc-Lys(Boc)-OH derivative, and Boc was removed with 50% TFA followed by the in situ neutralization coupling of Boc-Ser(*t*Bu)-OH with PyBOP.[26] Completion of the reaction was checked by Kaiser's test.[23] Further elongation revealed a sharp decrease in the substitution level suggesting a premature removal of N^{α}-Fmoc by the basic ε-amine in spite of the recommended in situ neutralisation coupling procedure.[27] In a second attempt, the lysyl residue was incorporated as Fmoc-Lys(Dde)-OH. After completion of the elongation with the last residue being N^{α}-Boc protected, the elimination of Dde by 2% N_2H_4 treatment in DMF was followed by Fmoc-Ser(*t*Bu)-OH coupling. UV measurement of the fluorenylmethyl-piperidine adduct revealed the incorporation of the seryl residue in 50–75% yield only. This fact could indicate a partial deprotection of the peptide chain from the resin by hydrazinolysis. Finally, we used the Fmoc-Lys(Mtt)-OH and removal of Mtt by treatment with 1% TFA in dichloromethane, but in the presence of Et_3SiH as a cation scavenger to avoid retritylation.[28] The yield corresponding to the removal of Trt and introduction of Fmoc-Ser(*t*Bu)-OH was estimated after piperidine treatment to be 90%. After deprotection of the side chain with TFA treatment followed by DIEA neutralization, the aminolysis with aminoacetaldehyde dimethylacetal was carried out to install, at the C-terminus, the aldehyde function protected as an acetal.[22b] HPLC and MS analyses revealed by-products



Scheme 1. Synthesis of the masked stem peptide dialdehyde Muc(Ser)acetal **4**: i) 1) HBTU (5 equiv), iPr$_2$NEt (6 equiv), 4 h; 2) DMF/Ac$_2$O/iPr$_2$NEt 2:2:1, 2 × 10 min; ii) 1) TFA/CH_2Cl_2 50:50, 30 min; 2) iPr$_2$NEt/NMP 1:9; 3) Fmoc/*t*Bu elongation; iii) 1) TFA/CH_2Cl_2/iPr$_3$SiH 1:98:1, ~30 × 2 min; 2) Fmoc-Ser(*t*Bu)-OH (5 equiv), HBTU (5 equiv), DIEA (6 equiv), 4 h; iv) piperidine/NMP 2:8; v) 1) TFA/H_2O/iPr$_3$SiH 95:2.5:2.5, 2 h; 2) iPr$_2$NEt/NMP 1:9; vi) H_2N-CH_2-$CH(OCH_3)_2$: DMF (2:1) 18 h, 40 °C.

with a Δm of $+96$ Da attributed to a trifluoroacetylation which were easily removed by treatment with piperidine.[29] The purity of the target peptide **4** with two masked aldehydes was estimated at 75% (Figure 2). The main impurity displayed a Δm of -163 Da. Considering that no drop in the UV recording of the fluorenylmethyl piperidine adduct was observed and that the elongation yield was 95%, we assumed that the by-product was due to the loss of the N-terminal dipeptide (Ala-Pro) during either the TFA treatment or the aminolysis step.

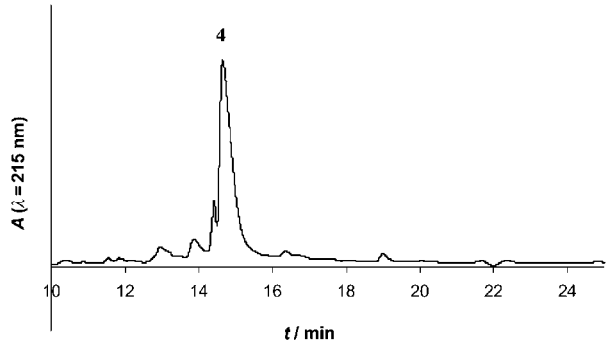


Figure 2. Analytical HPLC profile of Muc(Ser)acetal **4** (elution with gradient A).

With the stem peptide **4** in hand, two strategies for the construction of the final target **1** were envisioned: (Figure 3, pathway 1) after acetal deprotection and formation of the methyl-oxime bond with Aoa-PADRE **2**, the oxidation of the seryl residue was carried out followed by the formation of the keto-oxime bond with Aoa-Muc **3**; (Figure 3, pathway 2) inversely, the periodic oxidation first performed, followed by the ligation with Aoa-Muc **3** and, second, deprotection of the acetal followed by the ligation with Aoa-PADRE **2**. Based on preliminary results,[17] the former strategy has been explored. It was shown that the formation of the keto-oxime ligation was more sluggish than that of the methyl-oxime bond. As a likely consequence, transoximation of the methyl-oxime bond occurred during the formation of the keto-oxime. We thus decided to start the condensation of the building blocks by the keto-oxime bond formation (Scheme 2). Masked peptide dialdehyde **4** was oxidized by $NaIO_4$ to afford keto-aldehyde **5** which was then filtrated on a C18 Sep-Pak cartridge to remove the formaldehyde formed as a by-product during the reaction. The cartridge was copiously rinsed with water and **5** was eluted with

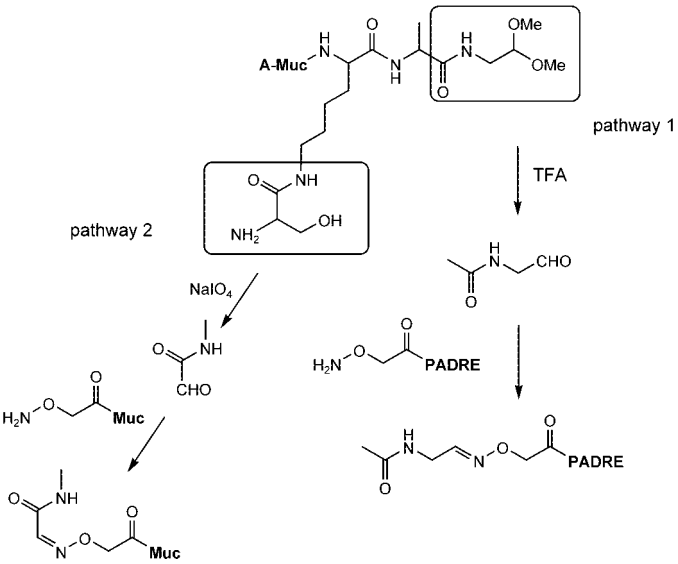
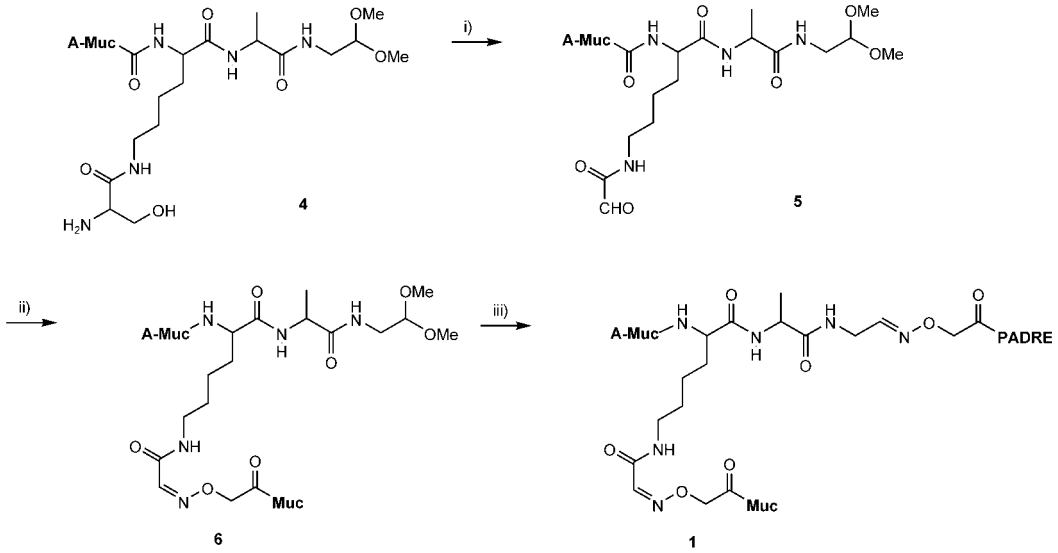


Figure 3. Possible pathways for successive unmasking of stem peptide dialdehyde **4**.



Scheme 2. Synthesis of the tri-branched peptide Muc(Muc)Padre **1**: double ligation strategy i) 1) $NaIO_4$, 2 equiv in 0.1 M phosphate buffer pH 6.5, 10 min; 2) HO-$(CH_2)_2$-OH, 2 equiv, 10 min; 3) Sep-Pak purification; ii) 1) Aoa-Muc **3**, 1 equiv in 0.1 M NaOAc pH 4.6, 18 h; 2) HPLC purification iv) 1) TFA/H_2O 60:40, 1 equiv Aoa-PADRE **4**, 6 min; 2) evaporation of TFA in vacuo; 3) 0.1 M NaOAc pH 4.6; 4) HPLC purification.

CH_3CN/H_2O (without TFA). After evaporation under vacuum, keto-aldehyde **5** was engaged in an oximation reaction with Aoa-Muc **3** in a 0.1 M NaOAc buffer, pH 4.6. The reaction was complete after 16 h and **6** was purified by HPLC.

After lyophilization, the acetal of **6** was removed by TFA treatment. HPLC analysis showed the presence of a by-product (40%) corresponding to Muc(Muc)-Muc. TFA deprotection led to a free aldehyde in the presence of a preformed keto-oxime bond. TFA-promoted reactivity of peptide aldehydes[30] probably induced the transoximation reaction as was reported for the levulinic acid[31a] and the pyruvic acid[31b] in acid medium. To circumvent the latter, we envisioned taking advantage of the TFA-promoted reactivity of the peptide aldehyde reasoning that the highly reactive aldehyde would preferentially react with Aoa-PADRE **2** if present in the medium during the TFA treatment. As partial deprotection of the acetal was observed during the purification–evaporation–lyophilization steps of **6**, compound **2** was added to the pool of HPLC fractions collected in an ice bath. After evaporation under vacuum and lyophilization, some of the final target compound **1** had already been present. The second ligation was completed after brief TFA treatment followed by evaporation under vacuum and addition of 0.1 M NaOAc up to pH 4.6.

Under these conditions, transoximation was prevented and the reaction was immediate and quantitative after addition of TFA. The target peptide **1** was eluted as a double peak corresponding to the *syn* and *anti* isomers of the oxime bond (Figure 4). They were well separated due to the very different and hydrophobic nature of the PADRE peptide when compared to the Muc peptide. The same procedure was followed for the synthesis of Muc(Muc)-Aoa **7** which contains just a simple Aoa residue instead of the Aoa-PADRE **2**.

After optimization of the ligation and purification steps, the overall yield of the tri-branched constructs ranged from 10% to 25%. The differences in yields were due to the last purification, depending on the hydrophobicity of the ligated compound. The use of the hydrophobic PADRE epitope in construct **1** gave a 10% overall yield whereas the use of Aoa gave Muc(Muc)-Aoa **7** in a 25% overall yield.

Evaluation of the immune response in mice: Mice were immunized with Muc(Muc)-PADRE **1** and the generated immune response was compared to that of Muc(Muc)-Aoa **7** (Figure 5). The monomeric peptide and the linear dimeric peptide with a C-terminal aldehyde engaged in an oxime bond with an Aoa residue, that is, Muc-Aoa **8** and MucMuc-Aoa **9**, respectively, were included in the trial. They did not induce the production of antibodies which recognized the MUC1 protein. This was expected since previous studies on MUC1 repeat units reported that at least four repeats were required to induce a measurable antibody response.[6] Interestingly, the presentation of the MUC1 units in a branched form, Muc(Muc)-Aoa **7**, with opposite orientations of the two monomers, stimulated the murine immune system to producing antibodies which strongly reacted with a recombinant unglycosylated MUC1 protein. Thus the different orientations of the monomers or the branching were sufficient to reduce the minimal requirement for the stimulation of antibody production from four repeats for the linear form to only two repeats in the branched form. The addition of the PADRE T-helper epitope to this short sequence of two MUC1-related antigens further increased the MUC1 specific humoral immune response in mice.

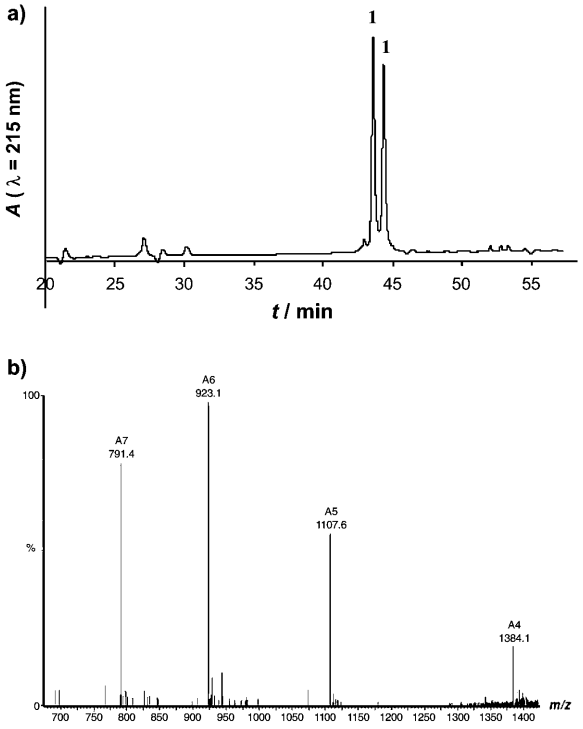


Figure 4. a) Analytical C18 RP-HPLC profile of Muc(Muc)PADRE **1** after purification. (gradient B). b) Mass spectrum of **1** analyzed by ESI-MS.

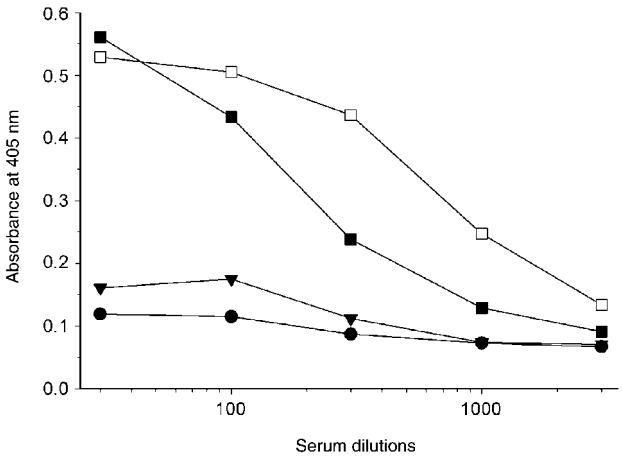


Figure 5. Serum antibody titers of mice immunized with MUC1 related peptides. C57Bl/6 mice were immunized subcuteanously with Muc-Aoa **8** (●), MucMuc-Aoa **9** (▼), Muc(Muc)-Aoa **7** (■) and Muc(Muc)-PADRE **1** (□). The mean values from three immunized mice are shown.

Conclusion

The results underline the strength of using chemoselective ligations based on oxime chemistry for the total chemical synthesis of the heteromeric tri-branched peptide construct. We have shown that a multi-ligation scheme based on successive unmaskings of the aldehyde group can be effective for the successive generations of the oxime bond. Besides the classical N-terminal specific ligation method, our ligation scheme employs the more challenging C-terminal ligation. We have also shown that our original tri-branched peptide construct is able to generate antibodies which recognize a recombinant MUC1 protein. These results open the way to new perspectives for the design of heteromeric branched peptides towards artificial proteins. The flexibility of this approach is being studied not only to test different universal T-helper epitopes but also to introduce Muc peptides diversely glycosylated in order to document the influence of the saccharide on the immune response against the tumor-associated MUC1.

Experimental Section

Abbreviations: dde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl, Fmoc: 9-fluorenylmethoxycarbonyl, GalNAc: 2-acetamido-2-deoxy-D-galactose, HATU: *O*-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt: 1-hydroxybenzotriazole, Mtt: 4-methyltrityl, PAM: phenylacetamidomethyl, PEGA: polyethylene glycol dimethylacrylamide co-polymer, PyBOP: 1-benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate.

General methods: Organic solvents were from SDS (Peypin, France) or Carlo Erba, with CH_2Cl_2, NMP, and piperidine being synthesis grade, and CH_3CN and MeOH being HPLC grade. Diethyl ether was from SDS (Peypin, France) and the higher purity from Acros (> 99.5). DMF was from Applied Biosystems (Courtaboeuf, France). TFA was from SDS (Peypin, France). Water was purified on a Milli-Q reagent system (Millipore). Boc-Ala-4-(oxymethyl)-phenylacetic acid linker was from Neosystem (Strasbourg, France). Fmoc-Rink-amide linker (*p*-{(*R*,*S*)-α-[1-(9*H*-Fluoren-9-nyl)-methoxyformamido]-2,4-dimethoxybenzyl}phenoxyacetic acid), Fmoc-Ala-Wang-resin and PEGA resin 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 et al.[32] 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 L7100 pump equipped with a C18 column, nucleosil 300 Å (5 μm, 250×4.6 mm) or a C18 column, Nucleosil 300 Å (5 μm, 250×10.5 mm) or a C4 column Vydac 300 Å (250×10.5 mm), a L-7455 diode array detector and a Merck-Hitachi interface D-7000. Peptides were eluted with a linear gradient of CH_3CN/H_2O/0.1 % TFA. Buffer A was water containing 0.1 % TFA, buffer B was CH_3CN containing 0.1 % TFA. Gradient A: 10–60 % of B over 80 min. Gradient B: 10 % of B for 10 min and 10–60 % of B over 80 min. Gradient C: 15 % of B for 6 min and 15–27 % of B over 30 min. The elution was followed at 215 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 a previous study.[33] We report herein only the data corresponding to the unfragmented peptides. MALDI-TOF mass spectrometry was performed on an Autoflex (Bruker).

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 at 0.1 mmol scale with HBTU/HOBt as coupling reagents. 10-Fold excess was used for protected amino acids and coupling reagents. The side-chain protecting groups used were Lys(Boc), Asp(O*t*Bu), Ser(*t*Bu), Thr(*t*Bu), His(Trt), Arg(Pbf), Trp(Boc). The 0.1 mmol scale program purchased from the manufacturer was used, with a single coupling followed by capping with acetic anhydride solution.

General procedure for manual coupling: Protected amino acid or linker (5 equiv) and HBTU (5 equiv) were dissolved in DMF. The solution was then transferred on resin (1 equiv) placed in a syringe equipped with a frit and $i\mathrm{Pr}_2\mathrm{NEt}$ (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 (3×).

Aoa-PADRE 2: H_2N-O-CH_2-CO-aKXVAAWTLKa-NH_2 (a: D-Ala; X: cyclohexylalanine): Fmoc-Rink linker (250 mg, 0.46 mmol) was manually coupled on an aminomethyl Tentagel resin (227 mg, 0.44 mmol g^{-1}) 2× 8 h. Elongation of the PADRE sequence was performed according to the general procedure with 78 % yield. Peptidyl-resin (110 mg, 0.021 mmol) was introduced in a syringe equipped with a frit and Boc-Aoa-OH (20 mg, 0.107 mmol) was manually introduced using the general procedure with HATU[34] (38 mg, 0.107 mmol) instead of HBTU. Peptidyl-resin (40 mg) was washed with CH_2Cl_2 (3×), and treated with TFA/H_2O/$i\mathrm{Pr}_3\mathrm{SiH}$ 95:2.5:2.5. Peptide was then precipitated and washed with ice-cold diethyl ether without carbonyl-containing compounds. The crude peptide (11 mg) was obtained. t_R (gradient B): 47.58 min; ESI-MS: *m*/*z*: calcd for $C_{64}H_{107}N_{17}O_{15}$: 1354.66; found: 1354.10 ± 0.19 $[M]^+$.

Aoa-Muc 3: H_2N-O-CH_2-CO-PPAHGVTSAPDTRPAPGST-OH: Compound **3** was prepared by using Fmoc-Ala-Wang-resin (250 mg, 0.44 mmol g^{-1}). Elongation (except for Aoa) was performed according to the general procedure in 95 % yield. Peptidyl-resin (240 mg, 0.052 mmol) was introduced in a syringe equipped with a frit and Boc-Aoa-OH (49.7 mg, 0.26 mmol) was manually introduced using the general procedure with HATU (99 mg, 0.26 mmol) instead of HBTU. Peptidyl-resin (41 mg) was washed with CH_2Cl_2 (3×), and treated with TFA/H_2O/$i\mathrm{Pr}_3\mathrm{SiH}$ 95:2.5:2.5. The peptide was then precipitated and washed with ice-cold diethyl ether without carbonyl-containing compounds. The crude peptide (14 mg) was obtained corresponding to a yield of 87 %. t_R (gradient A): 14.57 min; ESI-MS: *m*/*z*: calcd for $C_{82}H_{130}N_{26}O_{30}$: 1960.09; found: 1959.51 ± 0.06 $[M]^+$.

Muc(Ser)acetal 4: APPAHGVTSAPDTRPAPGSTAK(S)A-NH-CH_2-$CH(OCH_3)_2$: The Boc-Ala-4-(oxymethyl)phenylacetic acid linker (168.7 mg, 0.5 mmol) was manually coupled to the amino methyl PEGA resin (0.4 mmol g^{-1}; 4 g of wet resin) using the general procedure followed by capping with DMF/Ac_2O/$i\mathrm{Pr}_2\mathrm{NEt}$ 2:2:1 (2×10 min). The Boc-Ala-PAM-PEGA resin was deprotected with TFA/CH_2Cl_2 5:5 for 30 min. The Ala-PAM-PEGA resin was introduced into a reactor for solid-phase synthesis. The lysyl residue was introduced as Fmoc-Lys(Mtt)-OH. UV spectroscopy of the fluorenylmethyl-piperidine adduct at 301 nm (ε = 7800 $mol^{-1}dm^3cm^{-1}$) after removing the N^{α}-Fmoc gave the initial amount of amine available for elongation (0.076 mmol). The elongation of the A-Muc sequence was conducted as described in the general procedure. The N-terminal amino acid was introduced as Boc-Ala-OH. The dry peptidyl-resin (188 mg, 0.025 mmol) was introduced into a syringe equipped with a frit and treated with TFA/CH_2Cl_2/$i\mathrm{Pr}_3\mathrm{SiH}$ 1:98:1 (~30× 2 min, until the resin was colorless). Fmoc-Ser(*t*Bu)-OH (47.9 mg, 0.125 mmol) was manually coupled to the peptidyl resin using the general procedure. After three washings with NMP, the N^{α}-Fmoc of the serine was manually removed by 20 % piperidine in NMP (3×) followed by the washing of the resin with CH_2Cl_2 (3×). The absorbance of the fluorenylmethyl-piperidine adduct was measured at 301 nm (ε = 7800 $mol^{-1}dm^3cm^{-1}$) giving 0.021 mmol. The total elongation yield (backbone elongation and introduction of the branched serine) was 85 % starting from the initial amount of amine.

The Muc(Ser)PAM-PEGA resin was washed with CH_2Cl_2 (3×). The α-NH_2 and side chains were deprotected with TFA/H_2O/$i\mathrm{Pr}_3\mathrm{SiH}$ 95:2.5:2.5 for 2 h. The resin was washed with CH_2Cl_2 (3×) and NMP (3×) and neu-

tralized with *i*Pr_2NEt/NMP 1:9 followed by washings with DMF (3×). Aminoacetaldehyde-dimethylacetal (3.2 mL) and DMF (1.6 mL) were added to the peptidyl-resin and left under gentle stirring in an oven for 18 h at 40 °C. To recover the peptide acetal, the resin was drained and washed with DMF (3×) and H_2O (3×). The filtrates were pooled and evaporated under vacuum and, then, 20% piperidine in H_2O (1.2 mL) was added and left under stirring for 90 min. After evaporation under vacuum, the resulting oil was dissolved in distilled water (12 mL) and submitted to analytical C18-RP-HPLC. t_R (gradient A): 14.66 min; ESI-MS: *m*/*z*: calcd for $C_{99}H_{163}N_{31}O_{34}$: 2331.57; found: 2330.17 ± 0.66 $[M]^+$.

Muc(CHO)acetal 5 and Muc(Muc)acetal 6: Muc(Ser)acetal **4** (0.014 mmol) was dissolved in a 0.1 M phosphate buffer, pH 6.5 (8 mL). $NaIO_4$ (6.05 mg, 0.028 mmol) was added and the solution was left under stirring for 10 min. Ethane-diol (31.62 μL, 0.056 mmol) was added to quench the reaction. The peptide was semi-purified by filtration on C18 Sep-Pak. After evaporation under vacuum, Muc(CHO)acetal **5** was dissolved in 0.1 M NaOAc, pH 4.5 (4.8 mL) and submitted to C18-RP-HPLC. t_R (gradient A): 16.08 min; ESI-MS: *m*/*z*: calcd for $C_{98}H_{158}N_{30}O_{34}$: 2300.51; found: 2300.27 ± 1.09 $[M]^+$. Aoa-Muc **3** (30 mg, 0.015 mmol) was added to the solution of **5** and the reaction mixture was left under stirring for 18 h. Peptide conjugate **6** was purified by C18-RP-HPLC. HPLC fractions were kept in an ice bath, evaporated under vacuum and lyophilized in the presence of Aoa-PADRE **2** (3.3 mg, 0.0024 mmol). t_R (gradient A): 21.58 min; ESI-MS: *m*/*z*: calcd for $C_{180}H_{286}N_{56}O_{63}$: 4242.59; found: 4241.82 ± 0.64 $[M]^+$.

Muc(Muc)PADRE 1: Peptide acetal **6** in the presence of Aoa-PADRE **2** was treated with TFA/H_2O 4:6 (10 mL) for 6 min. After evaporation of the TFA under vacuum, the product was dissolved in 0.1 M NaOAc, pH 4.6 (6 mL) and immediately submitted to C18-RP-HPLC. Peptide **1** was purified by C4 RP-HPLC as a double peak and was recovered after lyophilization as a white powder (5.6 mg) with an overall yield of 10%. t_R (gradient B): double peak: 45.42/46.29 min; ESI-MS: *m*/*z*: calcd for $C_{242}H_{385}N_{73}O_{76}$: 5533.16; found: 5533.46 ± 0.45 $[M]^+$.

Muc(Muc)Aoa 7: Peptide acetal **6** in the presence of Aoa (1.4 mg) as treated with TFA/H_2O 4:6 (10 mL) for 6 min. After evaporation of the TFA under vacuum, the product was dissolved in 0.1 M NaOAc, pH 4.6 (6 mL) and immediately submitted to C18-RP-HPLC. Peptide **7** was purified by C18-RP-HPLC and was recovered after lyophilization with an overall yield of 25% (20.3 mg). t_R (gradient B): 30.01 min; ESI-MS: *m*/*z*: calcd for $C_{180}H_{283}N_{57}O_{64}$: 4269.52; found: 4267.67 ± 0.88 $[M]^+$.

Muc-Aoa 8 and MucMuc-Aoa 9: Muc-Aoa **8**: APPAHGVTSAPDTRPAPGSTAKA-NH-CH_2-CH=NO-CH_2-COOH; MucMuc-Aoa **9**: A(PPAHGVTSAPDTRPAPGSTA)$_2$-NH-CH_2-CH=NO-CH_2-COOH: After elongation, peptide-PAM-PEGA resin (0.024 mmol) was washed with CH_2Cl_2 (3×). The α-NH_2 and side chains were deprotected with TFA/H_2O/*i*Pr_3SiH 95:2.5:2.5 for 2 h. The resin was washed with CH_2Cl_2 (3×) and NMP (3×) and neutralized with *i*Pr_2NEt/NMP 1:9 followed by washings with DMF (3×). Aminoacetaldehyde-dimethylacetal (3.2 mL) and DMF (1.6 mL) were added to the peptidyl resin and left under gentle stirring in an oven for 18 h at 40 °C. The resin was drained and washed with DMF (3×) and H_2O (3×). Filtrates were pooled and evaporated under vacuum. The resulting oil was dissolved in distilled water (15 mL) and purified by C18-RP-HPLC. The peptide acetal (0.008 mmol) was then treated with TFA/H_2O 3:5 (8 mL) for 6 min. After evaporation under vacuum, the product was dissolved in 0.1 M NaOAc, pH 4.6 (10 mL). Aoa (3.5 mg, 0.018 mmol) was added and the solution was immediately submitted to C18-RP-HPLC. Muc-Aoa **8**: t_R (gradient A): 16.66 min; ESI-MS: *m*/*z*: calcd for $C_{96}H_{156}N_{31}O_{33}$: 2272.48; found: 2270.47 ± 0.15 $[M]^+$; MucMuc-Aoa **9**: t_R (gradient C): 26.5 min; ESI-MS: *m*/*z*: calcd for $C_{167}H_{263}N_{53}O_{58}$: 3941.25; found: 3942.24 ± 2.07 $[M]^+$.

Immunological tests: The immunological tests were carried out as described.[35] For each peptide, three C57Bl/6 mice were subcutaneously immunized with 100 μg of the peptide emulsified in incomplete Freund's adjuvant. The immunizations were repeated twice with two week intervals. Ten days after the last injection, blood was drawn from the retro-orbital vein and the antibody titers of the sera determined by ELISA on microtiter plates coated with unglycosylated recombinant MUC1 tandem repeats (160 aa). Goat anti-mouse IgG (H+L) conjugated to horse radish peroxidase (Bio-Rad, Marnes la Coquette, France) was used as secondary antibody and revealed by a colorimetric detection with 4-chloro-1-naphtol. All ELISA tests were performed in triplicate.

Acknowledgement

This work was supported by La ligue contre le cancer Région Centre, by a grant from the Centre National de la Recherche Scientifique: Protéomique et Génie des Protéines (FP and VP) and by the Groupement de Recherche: Génomique et Génie des Glycosyltransférases (FP and VP). One of us (GAC) would like to thank La Région Centre and CNRS for financial support (Bourse de docteur-ingénieur). We are also grateful to Mr Philippe Marceau for synthesizing the Muc-Muc-Aoa and to Ms Corinne Buré for recording most of the ESI-MS spectra.

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Received: July 29, 2004
Published online: November 5, 2004

