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Solid phase oxime ligations for the iterative synthesis of polypeptide conjugates

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Peptide-based complex biomacromolecules are now optimally assembled by sequential ligation of unprotected peptide segments. However, this approach is still limited by the laborious chromatographic purification and handling steps needed for multiple successive chemoselective couplings, which leads to loss of material. An efficient alternative is solid phase chemical ligation (SPCL) initially developed for native chemical ligation. We report here an extension of this approach to iterative oxime ligation reactions, and describe a streamlined approach for the modular preparation of oxime-containing polypeptides. In particular, we determined optimal conditions to remove the Alloc group in the presence of aminoxy and oxime ether groups, and we extended the applicability of iterative C-to-N SPCL through simplification of the access to a C-terminally-grafted, unprotected peptide segment, using solid supported chemical transformations only. The high purity of the crude oxime-containing polypeptides highlights the efficiency of our approach.

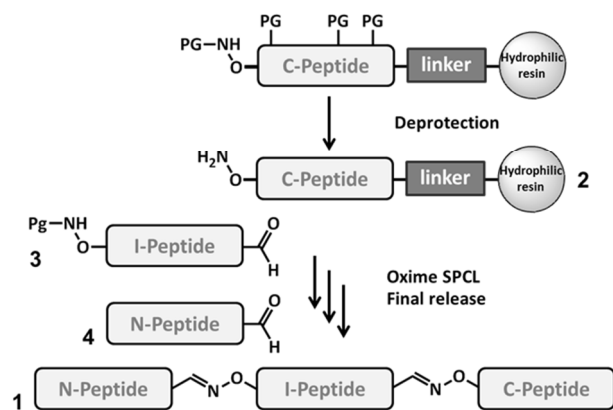
Introduction

The concept of solid-supported synthetic transformation was introduced by Merrifield half a century ago.¹ In the last two decades, the solid phase approach has been extended to chemoselective ligation reactions for the efficient construction of large biomacromolecules.²⁻⁵ As for the solid phase peptide synthesis (SPPS), the idea was to minimize the laborious chromatographic purification and handling steps needed for multiple successive chemoselective couplings, which leads to loss of material.⁶ The great potential of solid phase chemical ligation (SPCL),⁷ initially applied to native chemical ligation (NCL),² has been recently demonstrated for “click” copper-catalyzed and strain-promoted azide-alkyne cycloadditions, for the synthesis of large polypeptide⁴ and polynucleotide analogues,⁵ respectively. In line with our previous work focused on solid phase triazole⁴ and amide-forming^{3b} ligations, we thought of extending the repertoire of chemical ligation reactions operable on solid phase for the synthesis of large polypeptide conjugates.

“Click” oxime coupling of an aldehyde and an aminoxy group is an extensively utilized ligation reaction due to the high chemoselectivity of the process and its broad scope of applications.⁸ If examples of single solid supported oxime ligations have been reported, no iterative processes has yet been described, despite several existing iterative strategies for

solution phase synthesis of complex polyoxime biomacromolecular constructs.⁹

As starting material, peptide SPCL requires a naked peptide segment grafted onto a water-compatible solid support through a dedicated linker.¹⁰ The latter should be cleavable under mild conditions compatible with a large spectrum of peptides. In most cases,^{2,3b,4} an elaborate synthetic scheme was designed, including SPPS elongation, release of the properly functionalized naked peptide in solution followed by re-immobilization on a second (water-compatible) resin for subsequent elongation by successive ligations. The grafting of the naked segment at its N-terminus and the subsequent N-to-C elongation provides a self-purification effect.⁴ Alternatively, C-to-N SPCL elongation simplifies the overall synthetic scheme, and can be designed to exclusively include solid phase operations. Importantly, this strategy enables the use of the same resin for both SPPS and SPCL elongations. One report dedicated to iterative C-to-N solid phase NCL described such an approach, but it is restricted to the Cys-Gly dipeptide sequence as the first segment (*ie*, the C-peptide in scheme 1).^{3a} We thus decided to extend this strategy to trifunctional amino acids other than cysteine, focusing our synthetic goal on the elongation by iterative oximations for the construction of the model polypeptide **1** (scheme 1).



Scheme 1: Polypeptide synthesis through iterative oxime bond formation on solid support in the C-to-N direction

PG: protective group removable without affecting the TFA-cleavable linker. Pg: protective group removable without affecting the N-O bonds.

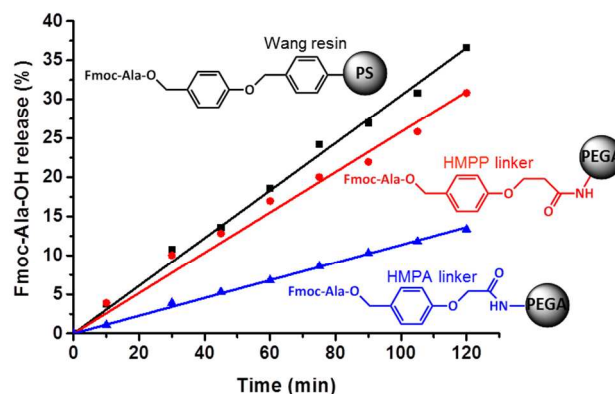
Results and discussion

The glycoprotein MUC1 was selected as our target. It is a highly glycosylated transmembrane protein with an extracellular domain, which is, to a large extent, made up of a 20 amino acid sequence (referred here to as [1-20]MUC1: PPAHGVTSPDTRPAPGSTA) repeated in tandem 30 to 150 times.¹¹ This is an ideal model to validate new chemical methodologies^{4,12} and a relevant biological target in tumor vaccine development.¹³ Oxime-containing compounds are particularly well suited for building original immunoconjugates, the oxime bond being sufficiently hydrolytically-stable¹⁴ to induce significant immune responses.^{8,13a,15}

Synthesis of naked C-terminal segment immobilized on a water-compatible resin 2 through an acid labile linker

Our strategy (scheme 1) relies on the starting material **2** which is composed of Meldal's polyethylene glycol polyacrylamide (PEGA) copolymer as a water-friendly solid support compatible both with SPPS and SPCL. To enable final peptide release using TFA, i.e., acidic conditions compatible with oxime bonds¹⁶ and with peptides bearing a large range of post-translational modifications, the linker should be stable to the chemoselective deprotection of the side chain protecting groups.¹⁷ Since the advent of the Fmoc/*t*Bu strategy in the development of SPPS, the 4-alkoxy benzyl ester linkers family (Wang-type) are the preferred linkers to cleanly release unprotected C-terminal peptide acids from the resin through a TFA treatment. As slight modifications on the phenol ether alkyl chain result in significant shifts in cleavage rates,¹⁸ our strategy required a re-examination of the stability of different Wang-type linkers when grafted to a PEGA resin. Two commercially available options were evaluated (scheme 2) for their stability under dilute TFA conditions (2% TFA) which are

considered to be sufficient for the removal of trityl-based protecting groups from side-chains (*vide infra*). As expected, we found the 4-(hydroxymethyl)phenoxypropionic acid (HMPP) linker was less stable than the 4-(hydroxymethyl)phenoxyacetic acid (HMPA) linker which behaved more similarly to Wang resin. Indeed, in HMPP the electron-withdrawing effect of the CONH on the phenolic oxygen is less pronounced than in HMPA (only one methylene as spacer in this case). Thus, the phenolic oxygen of HMPP is a better promoter of the acid lability of the benzyl ester in the para position. Similar results were obtained when these linkers were grafted to polystyrene resin.¹⁸ Accordingly, we employed the HMPA linker for the following steps towards the synthesis of **2** (see scheme 3).

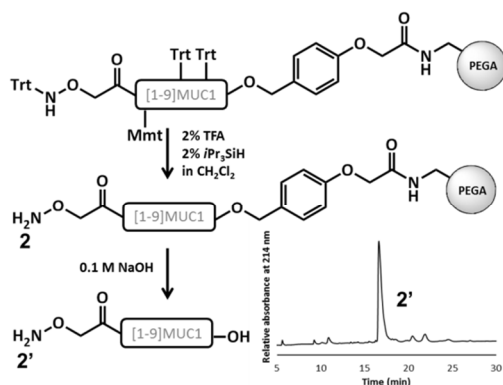


Scheme 2: Stability of Wang-type linkers to 2% TFA in CH₂Cl₂

The HMPA linker was expected to have sufficient stability to be compatible with a set of highly acid-labile protecting groups, all based on the trityl scaffold. Before employing them in SPCL, their acid lability was assessed in solution. Instead of the non-substituted trityl group, the highly acid-labile methoxytrityl (Mmt) group¹⁹ was chosen for the protection of the histidine imidazole. We found His(Mmt) to be quantitatively deprotected with 1% TFA in the presence of *i*Pr₃SiH as carbocation scavenger over 10 min. By comparison, unsubstituted trityl needs a 1-h treatment with 2% TFA to be quantitatively removed. Ser and Thr residues were protected by trityl ethers. The required *N*-Trityl protected aminoxycetic acid (Aoa) was prepared according to literature procedures.²⁰ A 15-min treatment with 1% TFA in CH₂Cl₂ was sufficient to quantitatively remove all trityl groups.

For the more challenging protection of the guanidine group of Arg, no highly acid labile protecting group has yet been described. We tested the use of ω,ω' -bis-allyloxycarbonyl (Aloc),²¹ which is removable using Pd(0) as a catalyst. However, we were unable to detect the expected peptide after SPPS using this building block. Rather, we observed multiple peaks having higher molecular mass than expected, consistent with loss of one of the Aloc group, and subsequent creation of branched peptides. Similar results were obtained with Fmoc-

Arg[*bis*(ω,δ)Aloc]-OH. Considering the position of Arg in our MUC1 sequence, we decided to explore the feasibility of our strategy using a shorter segment [1-9]MUC1 (PPAHGVTSA) (Scheme 3).



Scheme 3: Synthesis of supported C-terminal segment **2** [1-9]MUC1: PPAHGVTSA.

The PEGA resin loaded with an HMPA linker was esterified with Fmoc-Ala-OH following a described procedure.¹⁸ The SPPS elongation was carried out using HBTU/HOBt or HATU as coupling reagents. To completely remove the protecting groups, a continuous-flow treatment with 2% TFA in CH_2Cl_2 in the presence of 2% $i\text{Pr}_3\text{SiH}$ was applied until the yellow color due to the trityl carbocation was no longer visible. Characterization of peptide resin **2** required the release of peptide **2'** using a 0.1 M NaOH treatment, in order to avoid removing any remaining trityl groups. The HPLC trace revealed no peak exhibiting a UV spectrum related to the absorbance of trityl groups.

Synthesis of the internal segment **3**

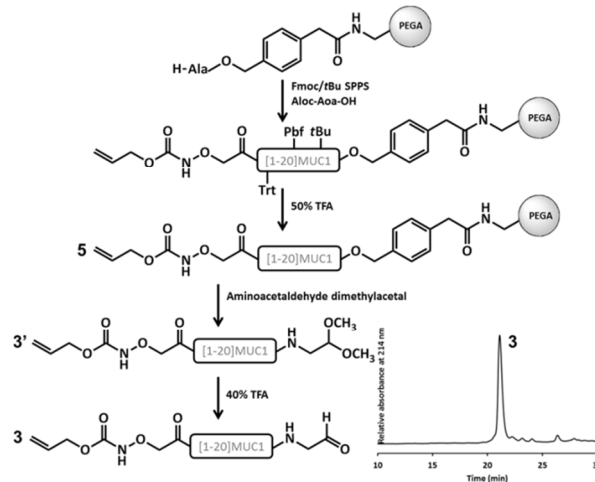
Another key component for our strategy is the internal peptide **3**. To prevent any oligomerization during oxime ligation, the N-terminal aminoxy function should be protected using a group removable after the first oximation, without affecting the oxime bond.

To install the aldehyde group at the C-terminus of **3**, we followed a procedure optimized in our laboratory (Scheme 4).²² An acetal-protected glycinal at the C-terminus is obtained by nucleophilic displacement of the ester between an unprotected peptide and the phenylacetamidomethyl (PAM) linker, with aminoacetaldehyde dimethylacetal. According to this procedure, the protecting group used to mask the N-terminal aminoxy function during oxime ligation should be stable under both acidic and basic conditions, that is, TFA treatment to remove the *t*Bu-based protecting groups from side chains as well as aminolysis with aminoacetaldehyde dimethylacetal; the *N*-Aloc group meets these requirements.²¹

The synthesis of Aloc-Aoa-[1-20]MUC1-CHO **3** began with the installation of the PAM linker on the PEGA resin using commercially available Boc-Ala-[4-(oxymethyl)phenylacetic acid]. After Boc removal by TFA treatment and neutralization with DIEA, the elongation was carried out following the Fmoc/*t*Bu strategy. *N*-Aloc-Aoa-OH was synthesized as previously described^{23a} and coupled with particular care so as to avoid overacylation of the nitrogen of the aminoxy group.²³ Subsequent cleavage of the acid-labile side-chain protecting groups were carried out with TFA/ $i\text{Pr}_3\text{SiH}/\text{H}_2\text{O}$. Finally, aminolysis through treatment with aminoethyl dimethylacetal furnished the internal segment **3'**. After HPLC purification, the segment was stored protected as the dimethylacetal until used for subsequent oxime ligations.

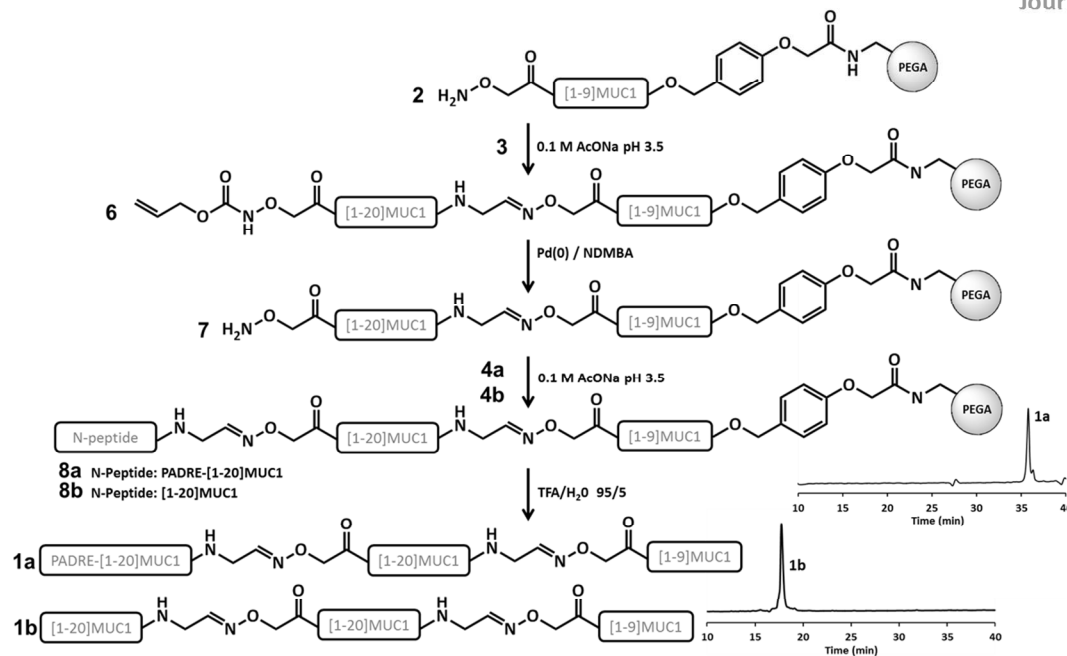
Synthesis of N-terminal segment aldehydes **4a** and **4b**

Two N-terminal segments were synthesized: **4a** ([1-20]MUC1-CHO) corresponding to one MUC1 repeat unit equipped with glycinal at the C-terminus and **4b**, where the PADRE (Pan DR Epitope) sequence, a highly efficient universal T-helper epitope, was elongated at the N-terminus of the MUC1 peptide to boost the immune response.^{15c} These peptide aldehydes were synthesized as described for the internal segment. Whereas the MUC1 sequence was known to be easily synthesized, the HPLC analysis revealed that crude acetal **4'a** was contaminated by *ca* 30% of a minor peak corresponding to the loss of 194 Da. It was attributed to the elimination of the two N-terminal proline residues during the aminolysis treatment, presumably by formation of a diketopiperazine.²⁴ To prevent this side reaction, the last N-terminal proline was introduced as an *N* α -Aloc-protected derivative.²⁵ It has to be noted that Aloc has to be removed using $\text{Pd}(\text{PPh}_3)_4/\text{dimethylamineborane}$ in the presence of boric acid to prevent the formation of allylamine.²⁶



Scheme 4: Synthesis of internal segment **3**

[1-20]MUC1: PPAHGVTSA PDTRPAGSTA.



Scheme 5 : Synthesis of oxime-containing MUC1 polypeptides **1** by iterative oxime SPCL.

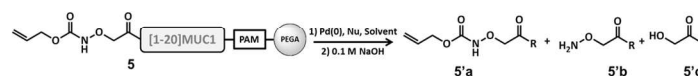
PADRE (pan DR epitope): aKXVAAWTLKAa, with a: D-Ala; X: cyclohexylalanine [1-20]MUC1: PPAHGVTSPDTRPAGGSTA. Insets: HPLC profile of crude polypeptides **1** after TFA release.

First oxime ligation followed by *N*-Aloc Aoa deprotection

Oxime “click” ligation can be carried out in water at moderately acidic pH, without the need for a catalyst, and typically without any degradation or unwanted side reactions,²⁷ provided that the aminoxy partners are kept away from carbonyl-containing compounds.²⁸ Accordingly, aminoxy peptide resin **2** was reacted with 1.5 equiv. of internal peptide aldehyde **3** at a 2 mM concentration (Scheme 5). The progress of the oxime ligation was followed by HPLC analysis of the supernatant at regular intervals. As soon as the amount of peptide aldehyde **3** remained constant (over 210 min and 300 min, at pH 3.6 and 4.6, respectively), the reaction was considered to be complete and was stopped by draining the supernatant and washing the resin. After extensive washes with H₂O and NMP, an aliquot of *N*α-Aloc peptide resin **6** was cleaved using TFA for quality control. The analysis of crude peptide **6** by HPLC and MS did not show the presence of any significant amount of starting material **2**, (see supplementary data). Note also the stability of the oxime bond under our final TFA treatment.

We anticipated that the conditions we classically use to remove the Aloc group could partially reduce the *N*-O bonds of **6** owing to the presence of a soft hydride donor as a nucleophile to scavenge the allylic cation. A careful optimization (Table 1) was thus undertaken using *N*α-Aloc-Aoa peptide resin **5**, a synthetic intermediate in the preparation of internal segment **3**.

As feared, when PhSiH₃ (Table 1, entry 1) and Me₂NH.BH₃ (Table 1, entries 2-7) were employed, partial cleavage of the *N*-O bond was observed. However, using two equiv. of Pd(PPh₃)₄ in the presence of the soft *C*-nucleophile *N,N*-dimethyl barbituric acid (NDMBA) for 30 min led to the quantitative removal of the Aloc group without affecting the aminoxy group (Table 1, entry 8). The latter conditions were applied to unmask the aminoxy of **6** to yield **7**. Gratifyingly, analytical HPLC of aminoxy peptide **7** showed that the *N*-Aloc removal was effective and the oxime bond remained intact.



	Solvent	Nu (equiv)	Pd(0) (equiv)	Time (min)	5'a (%) ^a	5'b (%) ^a	5'c (%) ^a
1	Anhydrous DMF ^b	PhSiH ₃ (50)	0.2	30	0	56	44
2	NMP ^c	Me ₂ NH.BH ₃ (6)	0.2	30	0	88	12
3	NMP ^c	Me ₂ NH.BH ₃ (6)	0.2	15	54	44	2
4	Anhydrous NMP ^d	Me ₂ NH.BH ₃ (6)	0.2	15	14	80	6
5	Anhydrous NMP ^d	Me ₂ NH.BH ₃ (6)	2	15	0	86	14
6	Anhydrous NMP ^d	Me ₂ NH.BH ₃ (6)	2	15	0	90	10
7	Anhydrous NMP ^d	Me ₂ NH.BH ₃ (6)	2	15	0	62	8
8	Anhydrous NMP ^d	NDMBA (6)	2	30	0	100	0

Table 1: Optimization of Aloc removal from *N*-Aloc with peptide resin **5**

a: calculated after integration of the HPLC-peak area; b: DMF distilled on Z-Gly-ONp; c: NMP used as received from commercially available sources; d: NMP kept on 4Å molecular sieves.

Second ligation followed by the release of the target peptide from the resin

Peptide resin **7** was finally engaged in the second oxime ligation with N-terminal peptide aldehydes **4a** and **4b** to afford polypeptide resins **8a** and **8b**, respectively. After releasing the peptides from the resin by TFA treatment, oxime-containing peptide **1a** and **1b** were obtained in good yields: 52% and 33%, respectively, starting from peptide resin **2**. The high purity of the crude target peptides (Scheme 5) enables us to use them for immunological applications even without HPLC-purifications.

Conclusions

In conclusion, we have extended the repertoire of chemical ligation reactions operable on solid phase for the synthesis of large polypeptide conjugates, introducing oxime SPCL. We have expanded the use of a simplified route of C-terminally grafted unprotected peptide segment by exploiting the chemoselectivity between a Wang-type linker for connecting the naked peptide segment to a water-compatible PEGA resin. We have also employed trityl-based scaffold protecting group strategy for the protection of trifunctional amino acids. During the iterative oxime SPCL process, the Aloc-group was used to protect the aminoxy moiety and its removal proved to be compatible with the presence of the N-O bond, either as an aminoxy or an oxime ether bond. To illustrate our modular approach, two final compounds were prepared from a common peptide resin. The high purity of the crude target peptides underlined the potential of SPCL for iterative oxime reactions as already observed for triazole ligation⁴ and amide-forming ligation.^{3b}

Experimental

Reagents and general experimental procedures

Unless stated otherwise, all reagents and solvents were used without further purification. Protected amino acids, Wang resin, PEGA⁸⁰⁰ resin, HMPA linker and HBTU were purchased from Merck Biosciences (Nottingham, UK). Boc-Ala-[4-(oxymethyl)-phenylacetic acid] and Fmoc-Ala-[4-(oxymethyl)-phenoxy-propan-3-oic acid] were purchased from polypeptide group (Strasbourg, France). Trt-Aoa-OH²⁰ and Aloc-Aoa-OH^{23a} were synthesized according to literature. Peptide synthesis grade DMF and HATU were obtained from Applied Biosystems (Courtaboeuf, France). Ultrapure water was prepared using a Milli-Q water system from Millipore (Molsheim, France). All other chemicals were from Sigma Aldrich (St-Quentin-Fallavier, France) and solvents from SDS-Carlo Erba (Val de Reuil, France). Diethyl ether free from carbonyl-containing compounds was from Acros (Geel, Belgium). Polypropylene syringes fitted with polypropylene frits were obtained from Torviq (Niles, MI, USA) and were equipped with PTFE stop-cocks bought from Chromoptic (Courtaboeuf, France).

Peptide characterization and purification

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 x 4.6 mm) or a C18 column, nucleosil 300 Å (5 µm, 250 x 10.5 mm), a L-7455 diode array detector and a Merck-Hitachi interface D-7000. Peptides were eluted with a linear gradient of MeCN in water. Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in MeCN. The elution was followed by absorbance at 214 nm. MALDI-TOF mass spectrometry was performed on an Autoflex instrument (Bruker) using α-cyano-4-hydroxycinnamic acid as the matrix. It has to be noted that the oxime bond fragments during a MALDI/TOF analysis, as we described earlier.²⁹ Here are reported only the molecular ions. The observed *m/z* correspond to the monoisotopic ions except when stated otherwise.

General procedure for automated solid phase synthesis

Solid phase peptide synthesis (SPPS) was run on a 433A automated synthesizer from Applied Biosystem using Fmoc/tBu chemistry on a 0.1 mmol scale with HBTU/HOBt as coupling reagent. A 10-equiv. excess of protected amino acids, HBTU and HOBt, and 20-equiv DIEA were used. The 0.1 mmol scale program (Fastmoc, SynthAssist™ version 2) was used with a single coupling followed by capping with acetic anhydride solution after each amino acid coupling. The instrument was coupled to a Perkin Elmer 200S UV/Vis detector to monitor the successive Fmoc deprotection.

General procedure for manual solid phase synthesis

Manual SPPS was performed on polypropylene syringes fitted with polypropylene frits (Torviq, Niles, MI, USA) equipped with PTFE taps (Chromoptic, Courtaboeuf, France). The resin or the peptide resin was swollen in DMF then drained. The protected amino acids, or linker, coupling reagents and DIEA were successively dissolved in DMF and the solution was transferred on the resin. The syringe was gently stirred for specified times. Excess reagents were eliminated by filtration and the peptide resin was washed successively with DMF (3x) and CH₂Cl₂ (3x).

Synthetic procedures for peptide resin 2

SYNTHESIS OF FMOC-ALA-HMPA-PEGA AND FMOC-ALA HMPP-PEGA FOR STABILITY TESTS

Fmoc-Ala-HMPA-PEGA: 4-Hydroxymethylphenoxyacetic acid (273.5 mg, 1.5 mmol, 3 equiv.) was coupled to aminomethyl PEGA resin (1.25 g dry resin, 1 equiv., 0.5 mmol) with HATU (570 mg, 1.5 mmol, 3 equiv.) and DIEA (520 µl, 3 mmol, 6 equiv.) dissolved in DMF (15 ml). The coupling was carried out for 2 h and the completion of the reaction was assessed by a qualitative Kaiser test. Then, to an ice-cold solution of Fmoc-Ala-OH (1.55 g, 5 mmoles, 10 equiv.) in dry DMF (40 ml) was added DCC (0.515 g, 0.25 mmol, 5 equiv.). After precipitation of dicyclohexylurea and filtration under a dry atmosphere, the filtrate was introduced in a fritted syringe containing dry HMPA-PEGA resin. Then, DMAP (6.1 mg, 0.05 mmol, 0.1

equiv.) solubilised in DMF (2 ml) was added and the syringe was stirred for 2 h followed by washings of the resin with DMF (3x), MeOH (3x) and CH₂Cl₂ (3x), successively. Finally, DMF/pyridine/Ac₂O (7/2/1) treatment for 30 min was performed to cap any unreacted hydroxyl groups. The yield over the two steps was estimated to 90% by UV titration ($\epsilon_{\lambda=301\text{nm}} = 7800 \text{ M}^{-1} \text{ cm}^{-1}$) of the Fmoc deprotection with a NMP/piperidine (4/1) treatment (3 x 3min) on an aliquot of dry Fmoc-Ala-HMPA-PEGA resin.

Fmoc-Ala-HMPP-PEGA: Fmoc-Ala-[4-(oxymethyl)-phenoxypropan-3-oic acid] (245 mg, 0.5 mmol, 5 equiv.) was coupled to the aminomethyl PEGA resin (0.25 g of dry resin, 1 equiv.) following the general procedure for manual solid phase synthesis with TBTU (190 mg, 0.5 mmol, 5equiv.) and DIEA (105 μl , 0.6 mmol, 6 equiv.) in DMF for 3h and the resin was washed with CH₂Cl₂ (3x), DIEA/CH₂Cl₂ (1/9, 3x), CH₂Cl₂ (3x). The completion of the reaction was checked by the qualitative Kaiser test.

Synthesis of peptide resin 2: Fmoc-Ala-HMPA-PEGA resin (0.1 mmoles) was introduced into a reactor and the elongation was conducted as described in the general procedure for automated SPPS using Thr(Trt), Ser(Trt), His(Mmt) as side chain protecting groups. After completion of the elongation, the peptide resin was transferred into a fritted syringe and Trt-Aoa-OH (165 mg, 0.5 mmol, 5 equiv.) was coupled following the general procedure for manual solid phase synthesis with HATU (190 mg, 0.5 mmol, 5 equiv.) and DIEA (105 μl , 0.6 mmol, 6 equiv.) in DMF for 2 h. Finally, very acid-labile protecting groups were removed by a continuous flow treatment with a CH₂Cl₂/TFA/*i*Pr₃SiH (96/2/2) solution for 10 min and the resin was washed with CH₂Cl₂ (3x), DMF (3x), CH₂Cl₂ (3x) and dried under vacuum.

To check the elongation and the removing of the side chain protecting groups, an aliquot of peptide resin **2** was treated with 0.2 M aqueous NaOH/DMF (1/1) for 30 min. The filtrate was neutralized with 5% aqueous TFA and peptide **2'** was analyzed by analytical C18-RP-HPLC and mass spectrometry. MALDI/TOF: [M+H]⁺ *m/z* = 909.3 (calcd for C₃₈H₆₁N₁₂O₁₄, 909.4). HPLC analytical gradient: 3-30% B/A over 30 min. Retention time: 16.3 min.

Synthetic procedure for internal segment 3

Boc-Ala-[4-(oxymethyl)phenylacetic acid] (168.7 mg, 0.5 mmol, 5 equiv.) was coupled to aminomethyl PEGA resin (0.25 g dry resin, 1 equiv.) following the general procedure for manual solid phase synthesis with TBTU (160 mg, 0.5 mmol, 5 equiv.) and DIEA (105 μl , 0.6 mmol, 6 equiv.) in DMF for 3h. The completion of the reaction was checked by the qualitative Kaiser test. The Boc protecting group was removed with TFA/CH₂Cl₂ (1/1) for 30 min and the resin was washed with CH₂Cl₂ (3x), DIEA/CH₂Cl₂ (1/9, 3x), CH₂Cl₂ (3x). The Ala-PAM-PEGA resin was transferred into a reactor and the elongation was conducted as described in the general procedure

for automated solid phase synthesis using Thr(*t*Bu), Ser(*t*Bu), His(Trt), Arg(Pbf) and Asp(O*t*Bu) as amino acid containing functional side chains. Then, the peptide resin was transferred into a fritted syringe. The N-terminal Aloc-Aoa-OH (70 mg, 0.4 mmol, 4 equiv.) was pre-activated (5 min) with DCC (82 mg, 0.4 mmol, 4 equiv.) and HOBT (54 mg, 0.4 mmol, 4 equiv.) in DMF and manually coupled for 2 h followed by successive washings of the resin with DMF (3x), MeOH (3x) and CH₂Cl₂ (3x). The completion of the reaction was checked by the qualitative chloranil test procedure. Side chain protective groups were removed with TFA/phenol/H₂O/*i*Pr₃SiH (88/5/5/2) for 1h and the resin was washed with CH₂Cl₂ (3x), DIEA/CH₂Cl₂ (1/9), and DMF (3x). Aminolysis was performed with a mixture of aminoacetaldehyde dimethylacetal and DMF (2/1) under gentle stirring for 18 h at 40°C. The resin was then drained and washed with DMF (3x). The filtrates containing acetal **3'** were pooled and evaporated under vacuum. The resulting oil was dissolved in buffer A and submitted to semi preparative C18-RP-HPLC. After lyophilization, acetal **3'** was obtained as a white powder in 69% yield, starting from Fmoc-Thr(*t*Bu)-Ala-PAM-PEGA determined after Fmoc removal and UV titration of the dibenzofulvene adduct at 300 nm ($\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$). Internal peptide acetal **3'**: MALDI-TOF: [M+H]⁺ *m/z* = 2131.1 (calcd for C₉₀H₁₄₄N₂₇O₃₃, 2131.0). HPLC analytical gradient: 3-39% B/A over 40 min. Retention time: 24.8 min. Just before oxime SPCL, aldehyde **3** was quantitatively generated through a treatment with TFA/H₂O (4/6) for 10 min. Internal peptide aldehyde **3**: MALDI-TOF: [M+H]⁺ *m/z* = 2085.0 (calcd for C₈₈H₁₃₈N₂₇O₃₂, 2085.0). HPLC analytical gradient: 10-35% B/A over 40 min. Retention time: 21.1 min.

Synthetic procedure for N terminal segments 4

PEPTIDE ALDEHYDE 4 (PADRE-[1-20]MUC1-CHO; PADRE (pan DR epitope): aKXVAAWTLKAa, with a: D-Ala; X: cyclohexylalanine)

The H-Ala-PAM-PEGA resin (0.1 mmol, prepared as described for internal segment **3**) was transferred into a reactor and the peptide elongation was conducted as described in the general procedure for automated solid phase synthesis using Thr(*t*Bu), Ser(*t*Bu), His(Trt), Arg(Pbf), Asp(O*t*Bu) as amino acid containing functional side chains and pseudoprolines as described.^{13a} Then, the peptide resin was transferred into a fritted syringe and the side chain protective groups were removed with TFA/phenol/H₂O/*i*Pr₃SiH (88/5/5/2) treatment for 2h followed by successive washings of the resin with CH₂Cl₂ (3x), DIEA/CH₂Cl₂ (1/9, 3x), CH₂Cl₂ (3x). Aminoacetaldehyde dimethylacetal in DMF (2/1) was added and left under gentle stirring for 18 h at 40°C. Then, the resin was drained and washed with DMF (3x). The filtrates were pooled, evaporated under vacuum and the resulting oil was dissolved in buffer A and submitted to semi-preparative C18-HPLC. After lyophilization, acetal **4'a** was obtained as a white powder in 65% yield, starting from Fmoc-Thr(*t*Bu)-Ala-PAM-PEGA resin (*vide supra*). MALDI-TOF: [M+H]⁺ *m/z* = 3236.5 (calcd for C₁₄₆H₂₃₈N₄₂O₄₁, 3237.0). HPLC analytical gradient :

10-35% B/A over 40 min. Retention time: 35.1 min. Just before oxime SPCL, aldehyde **4a** was quantitatively generated through a treatment with TFA/H₂O (4/6) for 10 min. Aldehyde **4a**: MALDI-TOF: [M+H]⁺ *m/z* = 3192.8 (calcd for C₁₄₄H₂₃₂N₄₁O₄₁ 3191.7). HPLC analytical gradient: 10-35% B/A over 40 min. Retention time: 32.2 min.

PEPTIDE ALDEHYDE **4b** ([1-20]MUC1-CHO)

The synthesis of acetal **4'b** was carried out as described for **4'a**, but the N-terminal proline was protected with an Aloc group. Aloc removal was performed as described.²⁵ Acetal **4'b** was purified by HPLC and recovered as a white powder in 68% yield, starting from Fmoc-Thr(*t*Bu)-Ala-PAM-PEGA resin. MALDI/TOF: [MH⁺]: *m/z* = 1973.3 (calcd for C₈₄H₁₃₇N₂₆O₂₉, 1974.0); HPLC analytical gradient: 10-35% B/A over 40 min. Retention time: 17.8 min.

Just before oxime SPCL, aldehyde **4b** was quantitatively generated through a treatment with TFA/H₂O (4/6) for 10 min. MALDI-TOF: [M+H]⁺ *m/z* = 1927.9 (calcd for C₈₂H₁₃₀N₂₆O₂₈, 1928.0). HPLC analytical gradient: 10-35% B/A over 40 min. Retention time: 13.0 min.

First oxime SPCL: Aoa-containing peptide resin **7**

Aldehyde **3** (9.5 mg, 4.5 μmoles, 1.2 equiv.) was dissolved in 0.1 M NaOAc, pH 3.6. The solution was introduced into a fritted syringe containing peptide resin **2** (3.75 μmol, 1 equiv.). The syringe was stirred gently. After 210 min, peptide resin **6** was washed with H₂O (3x), NMP (3x) and MeOH (3x) and dried under vacuum. An aliquot of **6** was treated with TFA/H₂O (95/5) for 2 h. Crude **6'** was precipitated in ice-cold Et₂O, washed with ice-cold diethyl ether, dried under vacuum, solubilized in buffer A and analyzed by HPLC and mass spectrometry. MALDI-TOF: [M+H]⁺ *m/z* = 2976.9 (calcd for C₁₂₆H₁₉₆N₃₉O₄₅, 2975.4). HPLC analytical gradient: 10-35% B/A over 40 min. Retention time: 23.9 min.

The N-terminal Aloc group of **6** was removed by treatment with Pd[P(Ph)₃]₄ (8.5 mg, 7.5 mmoles, 2 equiv.) and NDMBA (3.5 mg, 22.5 mmoles, 6 equiv.) in anhydrous NMP under argon atmosphere at 25°C for 30 min. Then, peptide resin **7** was washed with NMP (3x), CH₂Cl₂ (3x), MeOH (3x) and dried under vacuum. An aliquot of **7** was treated with TFA/H₂O (95/5) for 2 h. Crude **7'** was precipitated in ice-cold diethyl ether free from carbonyl-containing compounds,²⁷ washed with ice-cold diethyl ether, dried in vacuum, solubilized in buffer A, and analyzed by HPLC and mass spectrometry. MALDI-TOF: [M+H]⁺ *m/z* = 2892.6 (calcd for C₁₂₂H₁₉₂N₃₉O₄₃, 2891.4). HPLC analytical gradient: 3-39% B/A over 40 min. Retention time: 14.5 min.

Second oxime SPCL: Oxime-containing polypeptides **1a** and **1b**

Oxime reaction between aminoxy peptide resin **7** and peptide aldehydes **4a** or **4b** was carried out as described above for the first oxime SPCL. Peptide resins **8a** and **8b**, respectively, were

treated with TFA/H₂O (95/5) for 2 h. Crude conjugates **1a** and **1b** were precipitated in ice-cold Et₂O, washed with ice-cold diethyl ether, dried in vacuum, solubilized in buffer A and analyzed by HPLC and mass spectrometry. The crude conjugates **1a** (4 mg) and **1b** (5 mg) were obtained with a 52% and 33% yield respectively based on peptide resin **2**. **1a** MALDI-TOF: [M+2H]⁺ *m/z* = 3037.5 ([M] calcd for C₂₆₆H₄₂₀N₈₀O₈₃, 6064.1) average mass. HPLC analytical gradient: 10-35% B/A over 40 min. Retention time: 35.8 min. **1b** MALDI-TOF: [M+H]⁺ *m/z* = 4802.7 (calcd for C₂₀₄H₃₂₀N₆₅O₇₀, 4803.2), average mass. HPLC analytical gradient: 10-35% B/A over 40 min. Retention time: 17.3 min.

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Notes and references

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