



Synthesis of peptide di-aldehyde precursor for stepwise chemoselective ligations via oxime bonds

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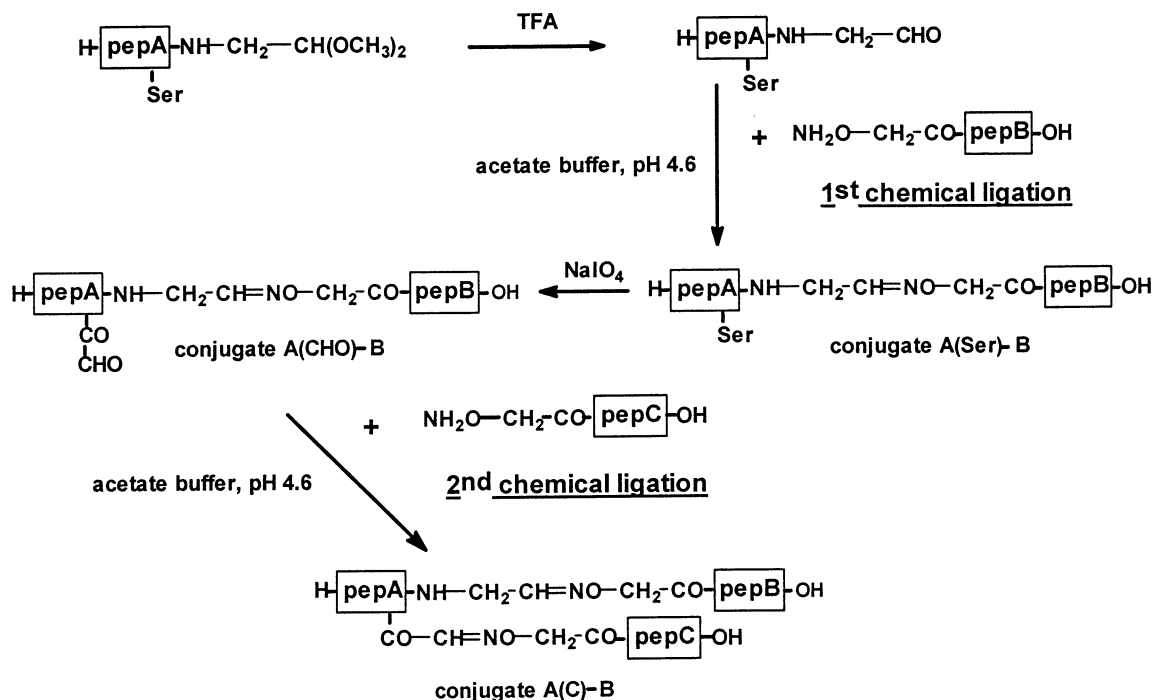
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Abstract—To synthesise a triple-function branched peptide in a modular way, we present a new strategy based on orthogonal generation of two aldehyde functions from an acetal and a 2-amino alcohol. Successive unmaskings of aldehyde functions of the stem peptide affords stepwise chemoselective ligations of two (aminooxy)acetyl peptides via oxime bonds. © 2000 Elsevier Science Ltd. All rights reserved.

Beside conventional protected segment condensation which is limited by poor solubility of protected peptides, chemoselective ligation has recently been developed for the synthesis of long, branched and cyclic

peptides.¹ This methodology relies on the ligation of unprotected peptides in aqueous buffer without any activated step thanks to the chemoselective coupling reaction of a C-electrophile on one peptide and a



Scheme 1. Modular synthesis of a tri-branched peptide through two successive chemoselective ligations.

Keywords: peptide aldehyde; chemoselective ligation; oxime; branched-peptide.

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N-nucleophile on another peptide. Depending on the organic reaction used, peptide-to-peptide ligations are achieved through formation of amide, oxime, hydrazone, thioester, thioether or thiazolidine bonds.^{1c} For most of the surrogate bonds, the C-electrophile partner is an aldehyde. It is classically obtained by periodate oxidation of a 2-amino alcohol (Thr or Ser) which could be incorporated at any position in a peptidic sequence through acylation of a selectively deprotected *N*^ε-lysine by a serine.² Alternatively, many studies have been devoted to the solid phase synthesis of peptide C-terminal aldehyde which could be obtained either free^{3–6} or masked.^{7,8} Among the latter, we have recently elaborated a strategy to synthesize a peptide with the aldehyde protected as an acetal.⁸ The key step was the cleavage of the ester bond between the peptide and the commercially available PAM⁹ resin by aminolysis with aminoacetaldehyde-dimethylacetal. In order to develop modular methods to synthesize triple-function branched peptides, we decided to explore the compatibility of our strategy⁸ with the generation of a second aldehyde by periodate oxidation.

We present here the completion of two stepwise orthogonal chemoselective ligations to an unprotected stem peptide. The modular strategy depicted in Scheme 1 is based on the successive generation of two aldehydes from an acetal and a 2-amino alcohol (Ser) borne on the stem peptide A. Stepwise chemoselective ligation of the (aminoxy)acetyl peptide B and the (aminoxy)acetyl peptide C affords the tri-branched chimeric peptide A(C)-B with two oxime bonds. To assess the potential of this approach, we synthesized a conjugate composed of the following model sequences:

- peptide A: H-Ala-Tyr-Asp-Ala-Lys(Ser)-Ala-NH-CH₂-CH(OCH₃)₂, so-called A(Ser)-CH(OCH₃)₂,
- peptide B: H₂NO-CH₂-CO-Asp-Ala-Arg-Ala-OH,
- peptide C: H₂NO-CH₂-CO-Trp-Lys-Gly-OH.

Peptide synthesis: The peptide A(Ser)-CH(OCH₃)₂ was synthesized using Boc-Ala-PAM-resin and Fmoc elongation. The lysine was introduced as Fmoc-Lys(Dde)-OH and the N-terminal alanine as Boc-Ala-OH. After complete elongation of the peptide, hydrazinolysis of the Dde group was followed by introduction of Boc-Ser(*t*Bu)-OH (Bachem, Switzerland) on the *N*^ε-lysine. TFA treatment^{10a} gave the unprotected peptidyl resin. Aminolysis of the ester bond between the peptide and the resin with aminoacetaldehyde-dimethylacetal⁸ (Aldrich) afforded the crude peptide A(Ser)-CH(OCH₃)₂ in 92% yield according to UV spectroscopy. The peptide A(Ser)-CH(OCH₃)₂ was then isolated in a pure form with a 78% yield following HPLC purification¹¹ and characterized by ESI-MS (calculated mass: 811.8, experimental mass: 811.8).

The (aminoxy)acetyl peptides B and C were assembled using conventional Fmoc/*t*Bu chemistry on a Wang resin. The side chain protecting groups were Arg, Pbf; Asp, *Or*Bu; Lys, Boc; Trp, Boc. Aminoxyacetic acid (Aoa) was introduced as Boc-Aoa-OH after being dissolved in DMSO, and the coupling was controlled by the Kaiser's test. TFA treatment^{10b} afforded the unpro-

tected peptides which were precipitated with diethyl ether. Diethyl ether has to be devoid of any trace of acetaldehyde as this quantitatively reacts with the aminoxy group to form the *N*-ethyloxime peptide. The (aminoxy)acetyl peptides B and C were purified to homogeneity by HPLC with a 62 and 67% overall yield, respectively, based on the resin substitution. They were characterized by ESI-MS (calculated mass: 504.5 and 462.5, experimental mass: 504.4 and 462.4 for (aminoxy)acetyl peptide B and (aminoxy)acetyl peptide C, respectively).

First ligation: The aldehyde of the peptide A(Ser)-CH(OCH₃)₂ (11.5 μmol, 1 equiv.) masked as an acetal was readily unprotected to afford the peptide A(Ser)-CHO with 95% TFA containing 5% H₂O at 20°C for 6 min.¹² After evaporation of TFA in vacuo and stabilisation of the pH at 4.6 with 0.1 M acetate buffer, the (aminoxy)acetyl peptide B (17.25 μmol, 1.5 equiv.) was added. The peptide aldehyde A(Ser)-CHO was totally depleted after 60 min and there was no significant formation of by-products. The conjugate A(Ser)-B was HPLC-purified with a 82% yield (experimental mass: 1251.9, calculated mass: 1252.3).

Second ligation: NaIO₄ oxidation of the *N*^ε-terminal serine of the conjugate A(Ser)-B provided a ketoaldehyde² without affecting the first oxime bond. The conjugate A(CHO)-B was isolated with a 52% yield after HPLC purification without special caution. To afford the second ligation, the conjugate A(CHO)-B (3.57 μmol, 1 equiv.) was reacted with the (aminoxy)acetyl peptide C (5.35 μmol, 1.5 equiv.) in 0.1 M sodium acetate, pH 4.6. The coupling reaction was followed by analytical HPLC. The desired conjugate A(C)-B (calculated mass: 1664.7, experimental mass: 1665.3) appeared rapidly as a double peak after 2 min. It took 24 h for the aldehyde partner (by default) to be depleted. During the time course of the reaction, a second double peak emerged which corresponds to the by-product A(C)-C (calculated mass: 1622.7, experimental mass: 1623.1). It increased up to 43%¹³ concomitantly with the decrease of the target conjugate A(C)-B. This is consistent with a transoximation reaction that likely occurs between the desired conjugate A(C)-B and the (aminoxy)acetyl peptide C, which was 1.5-fold in excess, leading to the conjugate A(C)-C. According to the kinetics, the target conjugate A(C)-B was maximum after 6 h (84%¹³), the conjugate A(C)-C being kept at 14%.¹³ The corresponding UV chromatogram is depicted in Fig. 1. Under these conditions, the conjugate A(C)-B was purified with a 52% yield. Starting from the peptide di-aldehyde precursor A(Ser)-CH(OCH₃)₂, the target conjugate was obtained with a 17% overall yield.

A third conjugate with a molecular mass of 1706.9 was attributed to the conjugate A(B)-B (calculated mass: 1706.7). It was stable during the time course of the reaction suggesting that it was formed before being mixed with the (aminoxy)acetyl peptide C. To verify this point, the peptide A(CHO)-B alone was left stirring in 0.1 M acetate buffer, pH 4.6. The presence of the conjugate A(B)-B was characterized in the reaction

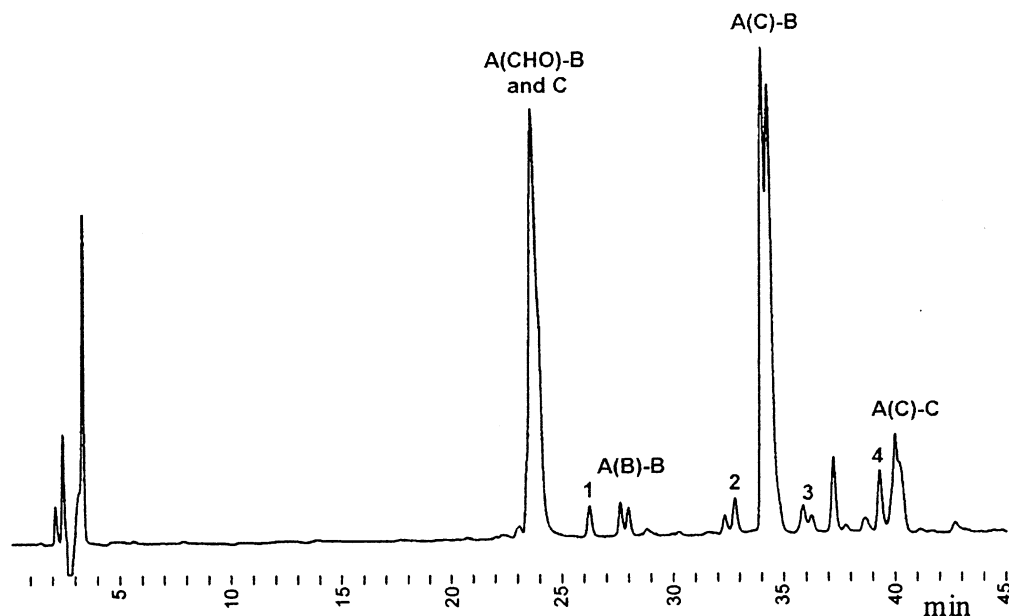


Figure 1. Analytical RP-HPLC of the second ligation mixture after 6 h at rt, C18 Lichrospher column (1 ml/min, linear gradient 3–30% acetonitrile in water, 0.1% TFA over 43 min).

mixture by HPLC and ESI-MS. This by-product would be the result of an intermolecular transoximation, with the aldehyde of the conjugate A(CHO)-B reacting on the oxime bond of another conjugate A(CHO)-B.

The formation of the by-products A(B)-B and A(C)-C underlines that transoximation reactions could occur when the methyloxime-containing peptide was in solution in the presence of either the aldehyde or the aminooxy partner. These side reactions were maintained at a minimum level. Moreover, it is worth stressing that oxime-containing conjugates studied in this work are quite stable upon storage at -20°C in the lyophilized state, even the conjugate A(CHO)-B.

As expected, the (aminooxy)acetyl peptide C is less stable.¹⁴ LC-MS/MS analysis of the purified (aminooxy)acetyl peptide C revealed that peaks 1, 2, 3 and 4 correspond to alterations of the peptide C at the level of the aminooxy group. Peak 1 (Fig. 1) corresponds to the hydroxyacetyl peptide C, probably formed by acidic hydrolysis of the N–O bond of the (aminooxy)acetyl peptide due to the presence of TFA in HPLC-collected fractions. Peaks 2, 3 and 4 correspond to compounds resulting from the addition of formaldehyde, acetaldehyde and acetone, respectively, to the aminooxy function of the peptide. These side products likely appeared during the last steps of purification or during storage. We precise here consequences of the reactivity of aminooxy compounds, briefly mentioned by others.¹⁴

Differences have been noted in the behaviour of the two oxime bonds handled in this study, i.e. methyl-oxime as the result of the first ligation and ketooxime as the result of the second ligation. Under our experi-

mental conditions, the methyloxime-forming ligation proceeded at a faster rate than the ketooxime. This is likely due to the α -substituent of aldehyde function, methyl and carbonyl, respectively. It could modulate the electrophilicity of the carbonyl. However, we cannot rule out that steric hindrance engendered by the longer peptide aldehyde A(CHO)-B as compared to the shorter one A(Ser)-CHO diminished the rate of oxime formation. Among all the ligation reactions realized in our laboratory, peptides containing a methyloxime exhibited a double peak on the HPLC traces, whereas peptides containing a ketooxime produced a single peak. This is consistent with the literature where *syn* and *anti* isomers of the oxime bond have been reported for conjugates composed of a sugar and an (aminooxy)acetyl peptide,¹⁵ or a steroid and an (aminooxy)alkylamine.¹⁶ To the best of our knowledge, such isomers have never been mentioned about conjugate containing a ketooxime.

In summary, we have developed a new strategy to efficiently achieve two stepwise chemoselective ligations for modular synthesis of tri-branched chimeric peptide. It proceeds under mild conditions in aqueous buffer and provides ligated compounds in good and reproducible yields. The successive generation of two aldehyde functions on a stem peptide offers a further level of orthogonality to chemoselective formation of oxime bonds beside those based on the NH_2 -protecting group chemistry for selective deprotection of aminooxy group.¹⁵ As an oxime bond is compatible with the immune response,¹⁷ we are currently applying this methodology for the preparation of a subunit vaccine composed of a universal T-helper, a B-cell and a T-cytotoxic epitopes needed to induce a complete immune response.

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9. Abbreviation used: Aoa: aminooxy acetic acid; Boc, *tert*-butyloxycarbonyl; DMSO, dimethylsulfoxide; ESI-MS: electrospray ionisation-mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; PAM, phenylacetamidomethyl; TFA, trifluoroacetic acid; TIS: triisopropylsilane.
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11. All peptides and conjugates were analysed and purified by analytical and semi-preparative RP 18 HPLC with a linear gradient 3–30% acetonitrile (0.1% TFA) in water (0.1% TFA) in 43 min. Characterizations were carried out by electrospray mass spectrometry.
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