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Simple and Efficient Solid-Phase Synthesis of Unprotected Peptide Aldehyde for Peptide Segment Ligation

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Abstract: We describe an efficient solid-phase synthesis of C-terminal peptide aldehyde. Making use of the stability of the PAM linker towards both acid and base conditions, a pentapeptide was synthesized starting from a PAM resin according to Fmoc/tBu chemistry. The side-chains were deprotected by TFA. The peptide was cleaved by aminolysis with aminoacetaldehyde-dimethylacetal leading to a C-terminal masked aldehyde. The unprotected peptide aldehyde was then coupled to amino-oxy derivatives by chemoselective ligation in aqueous solution.

Currently, the protein synthesis by chemical ligation of unprotected peptides is in great development[1]. Since Merrifield introduced SPPS, new synthetic protocols have continually been formulated toward greater efficiency in peptide assembly. Despite this progress, large peptides (with more than 50 residues) are difficult to synthesize by the stepwise solid-phase synthesis due to solubility problems and the accumulation of deletion /epimerization impurities. Chemical ligation of unprotected peptide fragments offers advantages over traditional coupling procedures by not requiring *in situ* activation of the carboxyl group nor side-chain protection; this results in high chemoselectivity and increased solubility. Futhermore, the chemical ligation has allowed incorporation of noncoded amino acids compared to the recombinant technology.

Oxime, hydrazone, thioester, thioether, thiazolidine or amides linkages have been produced by chemical ligation of unprotected peptide fragments. To be formed, most of these linkages have required an unprotected peptide aldehyde as the electrophilic partner. It has extensively been achieved by periodate oxydation of a terminal 1,2 aminoalcohol (Ser, Thr) to form a keto-aldehyde. Moreover, a few methods have been described for the solid–phase synthesis of C-terminal peptide aldehydes[2]. However, they required the synthesis of a special linker between the peptide and the resin, or/and a reductive treatment which is incompatible with Asp-, Glu-containing peptide. Here, we describe a simple solid-phase methodology to achieve a C-terminal peptide aldehyde, and its subsequent application for chemical ligation.

In the development of a methodology to produce peptide aldehyde directly from the solid phase, we made use of unique characteristics of the PAM-anchor originally developed for the Boc-SPPS[3]. The acid- and base-stable PAM anchor was chosen to allow the growth of the peptide *via* the Fmoc/tBu chemistry. After deprotection of the side chains by TFA, the peptide aldehyde was generated thanks to the sensitivity of the PAM-anchor to aminolysis[4]. The practicability of this strategy was tested through the synthesis of the Tyr-Asp-Ala-Lys-Ala-Gly-H pentapeptide aldehyde. This peptide aldehyde would then be joined to aminooxy derivatives (NH_2-O-CH_2-COOH and NH_2-O-CH_2-CO-AA-AA---) by chemoselective ligation *via* an oxime bond[5]. The general scheme of the synthesis of Tyr-Asp-Ala-Lys-Ala-Gly-H is depicted in scheme 1.

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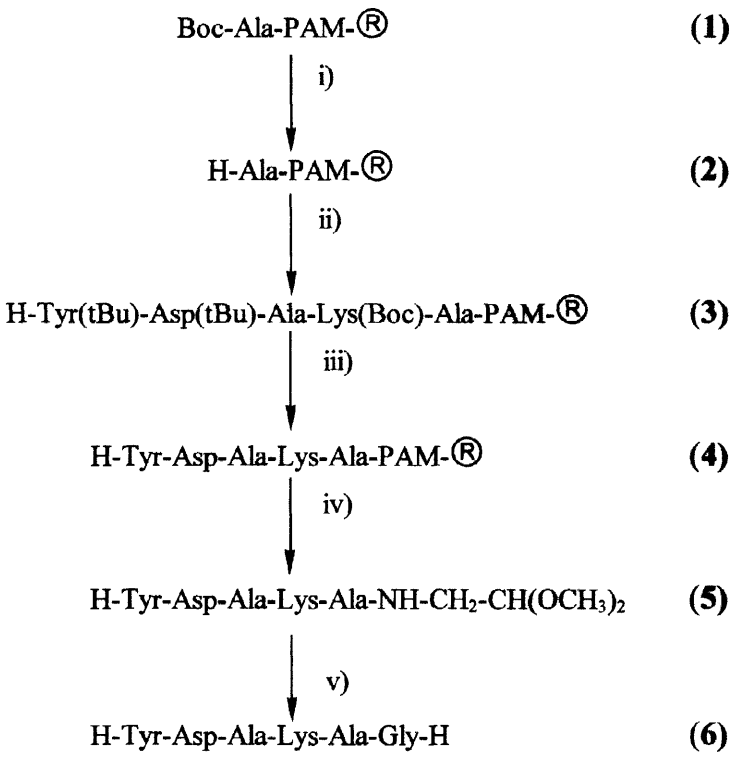
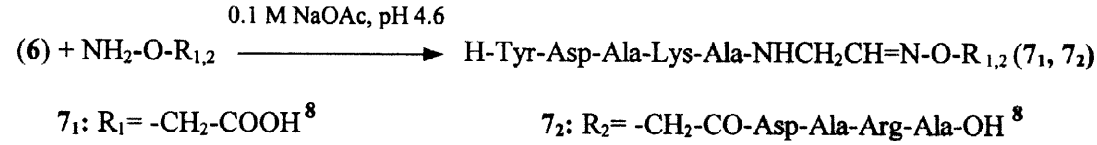


Scheme 1 : Stepwise synthesis of a C-terminal peptide aldehyde. i) TFA treatment followed by DIEA neutralization. ii) classical peptide chain elongation with Fmoc/tBu strategy. iii) TFA treatment. iv) aminolysis with aminoacetaldehyde-dimethylacetal. v) short TFA treatment.

The synthesis was carried out on an ABI 431 A peptide synthesizer with a marketing Boc-Ala- PAM resin (**1**) (Neosystem, France). After a classical Boc deprotection and neutralisation with standard module programme, the protected peptide-resin was synthesized using Fmoc/tBu chemistry with DCC /HOBt activation and single amino acid coupling. A 10-fold molar excess of activated amino acids was used for coupling. After releasing the Fmoc from the N-terminus, the deprotection of the side chains of (**3**) was achieved by a mixture of TFA/ CH_2Cl_2/ H_2O/ Phenol; 70:20:5:5 for 30 min. H_2O and Phenol were used as scavengers. To obtain (**5**), the peptide was cleaved from the resin (**4**) by aminolysis with aminoacetaldehyde-dimethylacetal (Aldrich). It has to be noted that the Pam linker is also susceptible to other N-nucleophiles such as dimethylamino-2-ethanol or ethanolamine[4]. After filtration of the resin, the wide excess of amine was evapored *in vacuo*. The oil was then solubilized in H_2O containing 0.1% of TFA. To establish optimal conditions for aminolysis, various protocols were tested including the use of different solvents (CH_2Cl_2, $CHCl_3$ or MeOH) and several temperatures (20°C or 45°C). The aminolysis product was characterized by analytical HPLC and electrospray mass spectrometry. Chloroform was used instead of dichloromethane, and the reaction was performed at 45°C. The best crude product in terms of yield and purity was obtained by treatment with aminoacetaldehyde-dimethylacetal, chloroform (2:3, v/v) at 45° C for 15 hours. The yield of the cleavage reaction was determined by UV spectroscopy ($\varepsilon_{274.8\ nm}$ of Tyr =1420), and was estimated at 98%.

The crude product (**5**) was then purified by semi-preparative reversed phase HPLC and the peptide-dimethylacetal was characterized by electrospray mass spectrometry (calculated mass: 653.71, experimental mass: 653.39) and ^1H NMR in DMSO-d_6. The presence of the two methoxy was characterized by the single peak at δ=3.24 ppm. The purified aminolysis product (**5**) was completely converted into the corresponding peptide aldehyde (**6**) by treatment with 95% TFA containing 5% H_2O at 20°C for 6 min[6] (figure 1).

To avoid any degradation and unwanted reactions of the aldehyde, the TFA was rapidly evapored, and the peptide aldehyde was mixed with the amino-oxy derivative (scheme 2) [7].



Scheme 2

The time course of the ligation reaction was monitored by analytical HPLC (Figure 2). Concomitantly with the decrease of the peak corresponding to the aldehyde, we observed the increase of two peaks corresponding to the conjugate. The presence of two peaks are likely due to the *syn-* and *anti-* forms of the oxime bond [9].

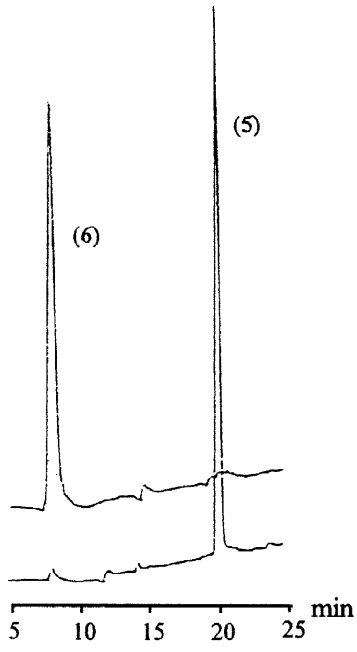


Figure 1
Analytical RP-HPLC profiles of Y-D-A-K-A-NH-CH_2-$CH(OCH_3)_2$ **(5)** and Y-D-A-K-A-NH-CH_2-CHO **(6)**

Same conditions as in figure 2.

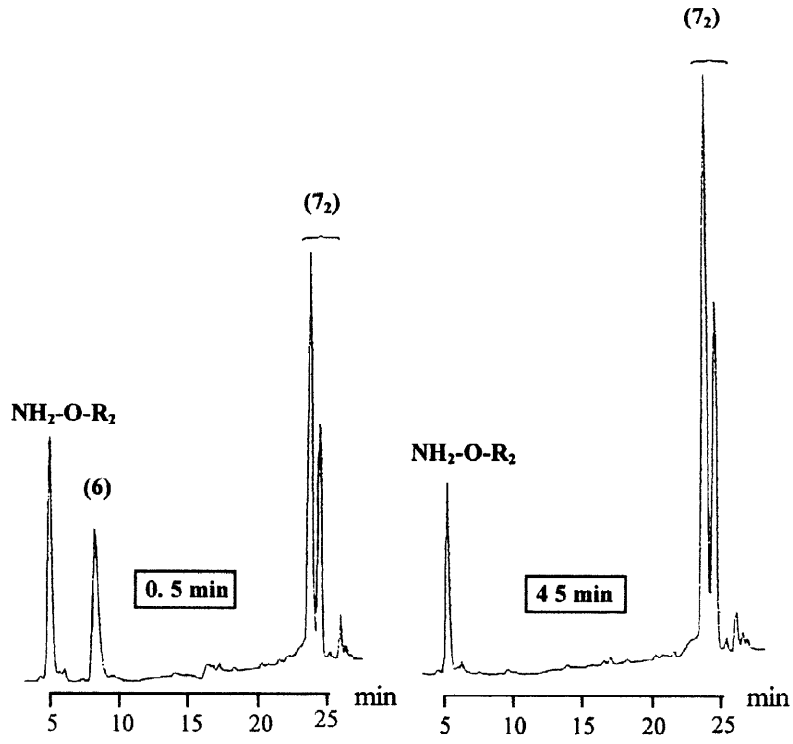


Figure 2
Analytical RP-HPLC profiles of the ligation reaction between **(6)** and NH_2-O-R_2 on a LiChrospher C_{18} column (4.5 x 250 mm).
Buffer A : 0.1 % TFA in water
Buffer B : 0.1 % TFA in CH_3CN/H_2O (60/40)
gradient : 10 % B for 5 min and 10 % B to 35 % B over 30 min.
UV monitoring at 214 nm, Flow rate 1 ml/min.

The reaction was very fast and finished in 5 min for (7_1) and in 45 min for (7_2) (Figure 2). The ligation product was purified by semi-preparative RP-HPLC and characterized by electrospray mass spectrometry (7_1: calculated mass = 681.61, experimental mass = 681.47; 7_2: calculated mass =1095.64, experimental mass =1095.29).

In summary, we have developed a very simple and efficient solid-phase synthesis of a C-terminal peptide aldehyde. The procedure did not require any synthesis of special linker, but the unique characteristics of the PAM-anchor, *i.e.* stability to both piperidine and TFA treatments and sensitivity to aminolysis. The peptide aldehyde was obtained in a very good yield (nearly 100 %). This opens the way towards a general value of this new approach. The presence of bulky residues, i.e valine and isoleucine at the C- terminus is now investigated as well as the synthesis of a large peptide-aldehyde (> 25 mer). The use of a masked aldehyde avoided difficult purifications due to the presence of free aldehyde. The protection of the aldehyde was removed just before performing the chemoselective ligation to the aminooxy partner. Moreover, the procedure demanded only cheap commercially available products. We are going to extend this methodology to the synthesis of modular chimeric peptides of immunological interest.

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References and notes

Abbreviation used : AA, amino acid; CH_3CN, acetonitrile; Boc, *ter*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; PAM, phenylacetamidomethyl; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid.

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7 Typical experiment of ligation : **solution A** : 0.92 mg (10^{-3} mmole, 1 eq) of **(5)** was treated with 200 µl of TFA containing 5 % H_2O at room temperature for 6 min. After removal of TFA, the released aldehyde was used right away for next step. **solution B** : 1.43 mg (2.10^{-3} mmole, 2 eq) of NH_2-O-CH_2-CO-Asp-Ala-Arg-Ala-OH was solubilized in 500 µl of 0.1 M sodium acetate, pH 4.6. **B** was added to **A** and stirred . The coupling reaction was followed by analytical HPLC.

8 NH_2-O-CH_2-COOH (7_1) was purchased to Sigma. NH_2-O-CH_2-CO-Asp-Ala-Arg-Ala-OH (7_2) was synthesized in our laboratory by Fmoc/tBu chemistry on a Wang-resin. Boc-NH-O-CH_2-COOH was coupled as an amino acid.

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