Pergamon

*Tetrahedron Letters*, Vol. 37, No. 7, pp. 933-936, 1996

0040-4039/96 $15.00 + 0.00

0040-4039(95)02394-1

# Acyl Disulfide-Mediated Intramolecular Acylation for Orthogonal Coupling Between Unprotected Peptide Segments. Mechanism and Application.

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**Abstract.** A highly efficient orthogonal coupling approach for peptide bond formation using unprotected peptide segments was described. The key element of this approach consisted of capturing an Npys modified N-Cys side-chain thiol of the amino segment with a $C^{\alpha}$-thiocarboxylic acid of the acyl segment to form an acyl disulfide which undergoes rapid intramolecular acylation to generate an amide bond. A final product with a native Cys residue at the ligation site was obtained after a thiolytic reduction step.

An important recent advance in peptide synthesis has been the development of orthogonal coupling strategies exploiting the proximity-driven intramolecular acylation for peptide bond formation,[1-3] a principle first put into practice by Kemp *et al.* who used a tricyclic dibenzofuran template to mediate the O,N-acyl transfer.[1] Intramolecular acylation overcomes the intrinsic entropic barrier of the intermolecular peptide bond-forming process for large segment condensation, confers high regio-specificity and orthogonality to the coupling reactions and avoids the need for protecting groups. Here, we describe a new, efficient, orthogonal coupling approach through acyl disulfide-mediated intramolecular acylation. Our method consists of the specific capture of an activated thiol side chain of an N-terminal Cys residue of the amino segment by the $C^{\alpha}$-thiocarboxylic acid of the acyl segment. This forms a mixed acyl disulfide which undergoes a rapid intramolecular S,N-acyl transfer through a 6-member ring intermediate. Thiolytic reduction of the resulting hydrodisulfide (S-SH) gives the native Cys residue at the ligation site (Fig. 1).

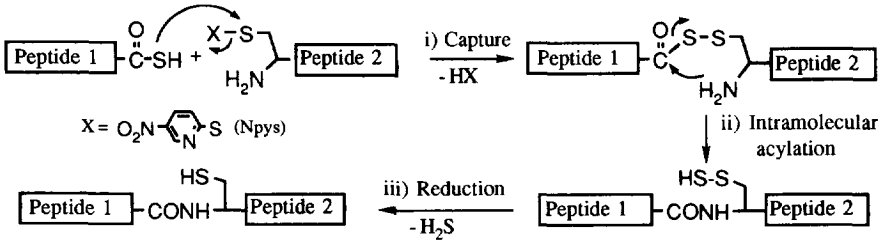


Fig.1. General scheme of the proposed orthogonal coupling method.

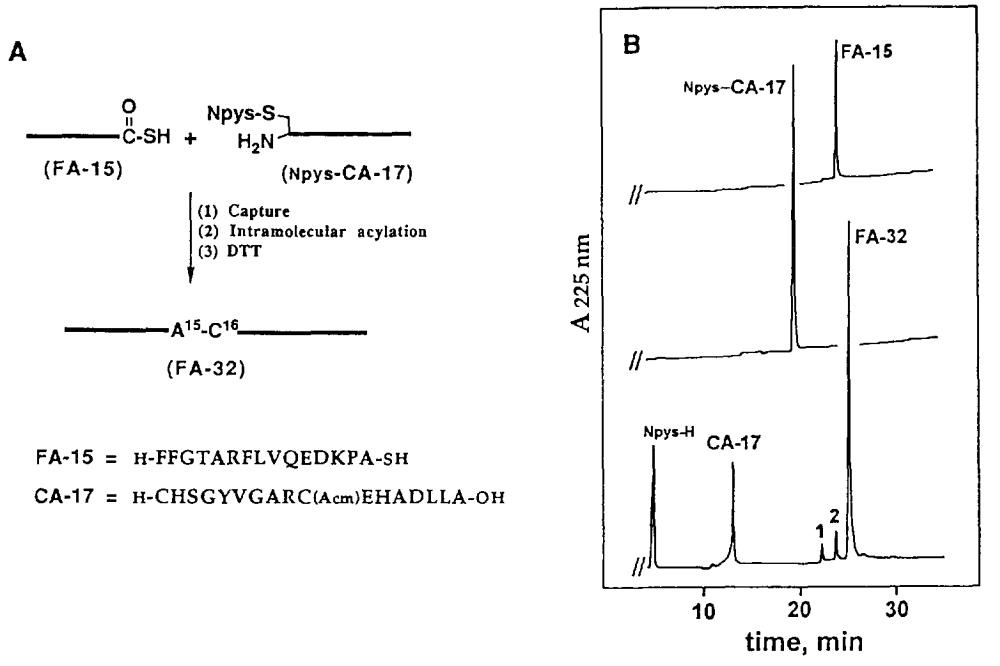


Fig. 2. A. Synthesis of a 32-residue peptide (FA-32) through ligation of FA-15 with Npys-CA-17. B. RP-HPLC monitoring of the synthesis of FA-32. Upper panel: FA-15; Middle panel: Npys-modified CA-17. Lower panel: DTT-treated ligation mixture after 5 min. Peak 1 was the hydrolyzed FA-15 with an α-COOH {[M+H]+, m/z 1726.2 found, 1726.0 calc.}; peak 2 was the remaining FA-15. HPLC was run on a Shimadzu system with a Vydac $C_{18}$ analytical column using a gradient of 20% to 55% Buffer B (60% $CH_3CN/H_2O$/0.04%TFA) in Buffer A ($H_2O$/0.045% TFA) for 35 min.

The efficiency and mechanism of this scheme was demonstrated by the synthesis of a 32-residue model peptide (FA-32) from two purified peptide segments (Fig. 2A), the acyl segment (FA-15) ([M+H]+, m/z 1743.3 found, 1743.0 calc.) containing a thiocarboxylic acid obtained by solid phase synthesis on a thioester benzhydryl resin[4] and the amine segment (Npys-CA-17) ([M+H]+, m/z 2027.0 found, 2027.3 calc.) containing an N-Cys(Npys) prepared from the reaction of free CA-17 with 2,2'-dithiobis(5-nitro-pyridine).[5] The experimental procedure consisted of mixing the two solutions (25% $CH_3CN$ in $H_2O$ with 0.05%TFA, pH 2) of FA-15 and Npys-CA-17 (1.2 fold excess) giving a final concentration of 10-15 μM. A yellow color developed immediately due to release of Npys-H, indicating occurrence of the capture reaction by sulfur-sulfur exchange. Solid sodium acetate was then added to adjust the pH to 6. After 5-10 min, the reaction mixture was treated with 1,4-dithiothreitol (DTT) to give the final product FA-32 (Fig. 2B) with expected MS ([M+H]+ 3582.2 found, 3582.0 calcd.). The high efficiency of the first capture step is attributed to the Npys-activated sulfide,[5] and to the super-nucleophilicity and low pKa value of the thiocarboxylic acid compared to a normal alkyl thiol. The acyl transfer was spontaneous and > 90% complete in 5 min as monitored by RP-HPLC. The efficiency of this acyl transfer step is attributed to the activated acyl disulfide[4b] and the proximity of the $C^{\alpha}$-acyl and $N^{\alpha}$-amine in a 6-member

ring intermediate (Fig. 1). The product of the intramolecular acyl transfer contained an S-SH group at the ligation site. Evidence supporting the S-SH product was unequivocal. Before DTT treatment, a product corresponding to S-SH was isolated and identified to have an additional 32-mass units (3614.5 found, 3614.0 calcd) compared to the final reduced product (FA-32). The S-SH was unstable and underwent disulfide exchange and oxidation reactions to give various tri- or tetrasulfide products as identified by MS. These included the trisulfide product formed between the S-SH product and the excess Npys-CA-17 as well as tri- and tetrasulfides formed between two S-SH molecules. The reduction step at the end of synthesis was indispensable to give the desired product FA-32 with a native Cys residue at the ligation site. The tri- and tetrasulfide byproducts had no effect on ligation yield since they were all converted to the desired Cys product upon reduction and a clean HPLC profile was obtained after the final reduction step (Fig. 2B, lower panel). Again, the release of $H_2S$ upon reduction provided other evidence supporting the involvement of S-SH product in our synthetic scheme. The purified final product (FA-32) gave a positive Ellman test[6] and contained the Ala-Cys peptide bond as analyzed by enzymatic digestion with thermolysin.

We have also studied an alternative scheme by first activating the thiocarboxylic acid as a mixed aryl disulfide as advocated by Yamashiro and Li[4b] and recently adopted by Dawson *et al.*[3] Activation of Z-Ala-SH with 5,5'-dithiobis(2-nitrobenzoic acid) [$(SNB)_2$, Ellman's reagent] yielded a mixed disulfide Z-Ala-S-SNB and not a 5-thio-2-nitrobenzoic acid ester (Z-Ala-SNB) as reported by Dawson *et al.*[3] Reaction of the mixed disulfide with a cysteinyl segment could proceed through two pathways (Fig. 3): (1) a sulfur-sulfur exchange with the N-Cys thiol to yield a covalent disulfide which undergoes S,N-acyl transfer through a 6-member ring intermediate as shown in Fig. 1 and (2) a direct coupling to the α-amine to yield a peptide bond.[4b] Since the reaction of Z-Ala-SSNB with Boc-Cys-Ala-OMe yielded Boc-Cys(Z-Ala-S)-Ala-OMe and the coupling of Boc-Ala-SSNB with H-Ala-OMe was slow, it is likely that the predominant pathway is (1). Nevertheless, a strategy of activating first the thiocarboxylic acid produces an enthalpically activated $C^{\alpha}$-acyl disulfide, which raises concerns of racemization and random acylation. These shortcomings are avoided in our proposed approach of activating the cysteinyl side chain in which an acyl disulfide is formed *in situ* and transforms rapidly to the stable amide bond. Indeed, no racemization was observed using a model reaction between Ac-Ala-Phe-SH and H-Cys(Npys)-Ala-OMe.[7]

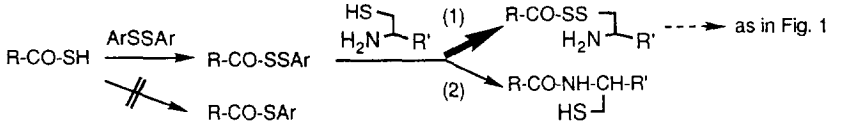


Fig. 3. Reaction pathways of an alternative activation strategy.

Starting from the capture step, our orthogonal coupling scheme is a one-pot process that does not require the isolation of intermediates. The high efficiency and simplicity makes this strategy an attractive approach for the synthesis of proteins involving very large unprotected peptide segments under relatively low molar concentrations. This scheme is conceptually similar to the thiol-capture strategy of Kemp,[1] the thiol-thioester exchange[8] and the

aldehyde-capture[2] orthogonal coupling methods developed in our laboratory. Such orthogonal coupling approaches combine the benefits of the solid phase synthesis[9] for its efficiency in the preparation of large synthetic segments and the solution segment condensation for using purified segments to avoid ambiguity. They hold exceptional promise for the synthesis of large native and artificial proteins.

**Acknowledgement:** This work was supported by US PHS grants CA 36544 and AI 37965.

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7. Ac-Ala-Phe-SH was reacted with Tfa. H-Cys(Npys)Ala-OMe and the product was analyzed by reverse phase HPLC which revealed no Ac-Ala-D-Phe-Cys-Ala-OMe for which the retention time was known from an authentic product.

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(*Received in USA* 9 *October* 1995; *accepted* 1 *December* 1995)