ORGANIC LETTERS

2004
Vol. 6, No. 26
4861−4864

# Native Chemical Ligation through in Situ O to S Acyl Shift

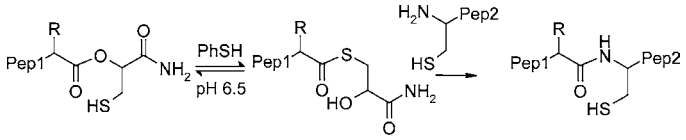
**Paolo Botti,* Matteo Villain, Sonia Manganiello, and Hubert Gaertner**

*Geneprot Inc., Geneva Branch, 2, Pre-de-la Fontaine, 1217 Meyrin, Switzerland*

*paolo.botti@geneprot.com*

**Received September 17, 2004**

## ABSTRACT




**A novel strategy to generate thioester peptides compatible with Fmoc chemistry is presented. Peptide-C$^{\alpha}$oxy-(2-mercapto-1-carboxyamide)-ethyl ester undergoes an O to S acyl shift during ligation and the newly formed thioester intermediate reacts with an N-terminal cysteine fragment generating a product with native amide bond at the ligation site.**

Native chemical ligation (NCL)[1] has been extensively demonstrated to be the key to the rapid preparation of small- and medium-size proteins with a high level of homogeneity.[2]

Such methodology exploits a chemoselective reaction between two unprotected fragments, a C-terminal thioester and a N-terminal cysteine, in aqueous solution at neutral pH. In the first step of this reaction, transthioesterification occurs at pH 6−8 by thiol exchange between the free thiol of the N-terminal cysteine and the thioester moiety on the other molecule. The newly generated thioester then undergoes an S to N acyl shift due to the proximity of the amino group to the thioester functionality, thus generating a native amide bond at the ligation site. Presently, the synthesis of the oligopeptide thioester intermediates is carried out primarily by Boc-based chemistry and less so by Fmoc-based chemistry. This is due to the instability of the thioester groups to the nucleophilic reagents used to remove the protecting groups in Fmoc-like approaches. On the other hand, the extremely strong acidic conditions, like HF or TFMSA used in Boc SPPS, are generally incompatible with post-translational modifications (PTM), which are often necessary for biological activity. It is then clear that a simple and practical strategy, which allows the generation of thioester fragments under conditions compatible with PTM derivatizations, is essential for the preparation of polypeptides with high biological value.

Several attempts have been made to overcome this problem: the Bertozzi and Pessi groups[3a,b] first proposed the use of a safety catch linker developed by Ellman[4] on the original Kenner's sulfonamide[5] linker that must be activated by alkylation prior to cleavage. A novel BAL linker strategy has been developed by Barany[6] to generate peptide thioesters via Fmoc chemistry, but also in this case some additional post-chain assembly manipulations are required. Lately, Clippingdale et al.[7] utilized DBU as a non-nucleophilic base to remove the Fmoc group; this reduced, but not completely abolished, thioester degradation. Brask et al. then developed a novel and interesting trithioortho ester masking strategy using a BAL linker; however, its practical utility at the present is restricted mainly to the preparation of glycine thioester peptides.[8] Very recently, to generate peptide

(1) Dawson P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.
(2) (a) Dawson P. E.; Kent S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923−960. (b) Kochendoerfer G. G. et al. *Science* **2003**, *299*, 884−887. (c) Rose, K. et al. *Proteomics* **2004**, *4*, 2125−2150.
(3) (a) Shin Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684−11689. (b) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369−11375.
(4) Backes, B. J.; Virgilio, A. A.; Ellman, J. A. *J. Am. Chem. Soc.* **1996**, *118*, 3055−3056.
(5) Kenner, G. W.; McDermott, J. R.; Sheppard, R. C. *Chem. Commun.* **1971**, 636−637.
(6) Alsina, J.; Yokum, T. S.; Albericio, F.; Barany, G. *J. Org. Chem.* **1999**, *64*, 8761−8769.
(7) Clippingdale, A. B.;Barrow, C. J.; Wade, J. D. *J. Pept. Sci.* **2000**, *6*, 225−234.

10.1021/ol0481028 CCC: $27.50 
**Published on Web 11/19/2004**

thioesters Camarero et al.[9] utilized an aryl hydrazine linker, which nevertheless requires an oxidation step after chain assembly completion of the peptide of interest. Accordingly, a simple, general, and practical way to generate thioester fragments via Fmoc chemistry is needed. Unlike thioesters, carboxyesters are widely used in Fmoc chemistry to link the peptide chain to the solid support due to their stability under the conditions required to remove the Fmoc group[10] even when the cleavage of the peptide from the linker is performed under basic conditions.[11] The aim of our work is to design a strategy to generate peptide-C$^{\alpha}$thioesters via standard Fmoc chemistry that does not require any additional step after the first FmocAA is coupled to the appropriate resin. To fulfill these requirements the peptide must be assembled onto a O-linked template that is stable to (i) Fmoc deprotection cycles and (ii) TFA-like cleavage and (iii) allows the generation of the thioester species prior to or during the ligation reaction. In summary, if the first FmocAA of the sequence is attached to the template through a carboxyester linkage, a routine Fmoc SPPS synthesis and cleavage should be performed. Only prior to or during ligation does the generation of the thioester moiety occur using standard NCL reaction conditions.[12]

In this paper, we present a new methodology to obtain thioester intermediates through in situ O to S acyl shift (Figure 1). We envisaged that a peptide-C$^{\alpha}$carboxyethylester

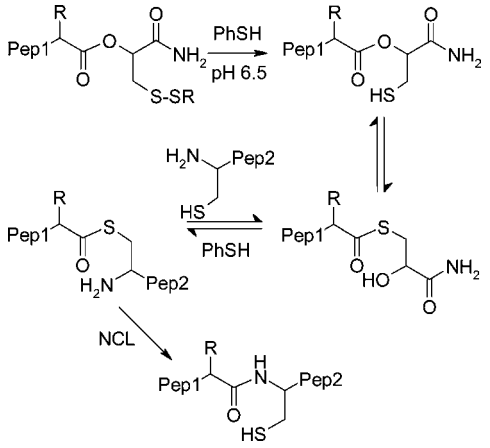
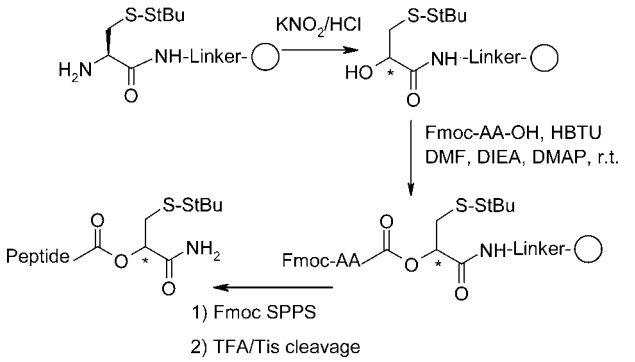



**Figure 1.** Native chemical ligation through in situ O to S acyl shift.

bearing a free mercaptan in the $\beta$ position might be in an equilibrium with its thioester isomer through an O to S acyl shift via a five-member ring intermediate. Our hypothesis found some support in early work concerning the stability of thiol esters with a hydroxyl group in the $\beta$ or $\gamma$ position,[13] even if such work indicated that the equilibrium between the two forms favored oxygen ester formation. It is known that the sulfhydryl group of a mercaptan is more acidic than the hydroxyl group of the corresponding alcohol. Furthermore, mercaptans can form salts in aqueous alkali and $H_2S$ is an acid much stronger than $H_2O$.[14]

Therefore, at the pH required for native chemical ligation the sulfhydryl group is more dissociated than its corresponding hydroxyl isomer making the O to S acyl equilibrium unfavorable. If, however, an electron-withdrawing group is introduced adjacent to the oxygen occupied in the ester bond, activation of the carboxyester occurs making the hydroxyl moiety a better leaving group and allowing a more favorable O to S acyl equilibrium between the two isomers. In this strategy a peptide fragment C$^{\alpha}$carboxyester of the 3-*tert*-butyldisulfanyl-2-hydroxypropionamide (Scheme 1) is synthesized using standard Fmoc chemistry and is cleaved after chain assembly under standard TFA conditions (TFA/$H_2O$/TIS 95/2.5/2.5% v/v) at rt for 1 h. The preparation of peptide-C$^{\alpha}$oxy-(2-*tert*-butyldisulfanyl-1-carboxyamide)ethyl ester is described in Scheme 1. Fmoc-Cys(tButhio)-OH is coupled to amino-Rink-PEGA resin using standard HBTU coupling. After Fmoc removal with 20% piperidine, the N-terminal free amino group reacted with $KNO_2$ under aqueous acidic conditions[15,16] to generate the 3-*tert*-butyldisulfanyl-2-hydroxypropionamide template as a racemic mixture.

**Scheme 1.** Preparation of Peptide-C$^{\alpha}$oxy-(2-*tert*-butyldisulfanyl-1-carboxyamide) Ethyl Ester Starting from Rink Amide PEGA Resin




The first amino acid of the sequence is attached to the template via HBTU-mediated coupling with DMAP as

(8) Brask, J.; Albericio, F.; Jensen, K. J. *Org. Lett.* **2003**, *5* (16), 2951−2953.
(9) Camarero, J. A.; Hackel, B. J.; de Yorero, J. J.; Mitchell, A. R. *J. Org. Chem.* **2004**, *69*, 4145−4151.
(10) Atherton, E.; Logan, C. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1980**, 538−546.
(11) Martinez, J.; Laur, J.; Castro, B. *Tetrahedron Lett.* **1983**, *24*, 5219−5222.
(12) (a) Dawson, P. E.; Churchill, M. J.; Ghadiri, M. R.; Kent, S. B. H. *J. Am. Chem. Soc.* **1997**, *119*, 4325−4329, (b) Tam, J. P.; Lu, Y. A.; Liu C. F.; Shao J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485−12489. (c) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10068−10073.
(13) (a) Harding, J. S.; Owen, L. N. *J. Chem. Soc.* **1954**, 1528−1536. (b) Harding, J. S.; Owen, L. N. *J. Chem. Soc.* **1954**, 1536−1545. (c) Jenks, W. P.; Cordes, S.; Carriuolo, J. *J. Biol. Chem.* **1960**, *235*, 3608−3614.
(14) Lide, D. R. In *Handbook of Chemistry and Physics*, 81st ed.; CRC Press: Boca Raton, 2000−01; Vol. 8, pp 44−46.
(15) Harris, C. M.; Schneider, M. J.; Ungemach, F. S.; Hill, J. E.; Harris, T. M. *J. Am. Chem. Soc.* **1988**, *110*, 940−949.
(16) Typically, the reaction with $KNO_2$ is performed in diluted HCl (0.2−0.5 M) depending on the different batch and substitution of the PEGA resin. Optimal conditions may vary from batch to batch of resin.

catalyst (0.1 equiv to the theoretical resin loading) for 1 h. The first residue is then coupled a second time under identical conditions (double coupling) to ensure complete esterification of the hydroxyl moiety. The overall yields of these reactions are approximately 45% as determined by Fmoc release UV assay.

Esterification of a secondary alcohol is generally difficult, and in order to maximize the yield, the attachment of the first residue is optimized using as a model study the coupling of Fmoc-Phe to a polymer-bound phenyllactic acid. On such models, we compared the coupling using DCC via symmetrical anhydride with the HBTU/DIEA activation, both with DMAP as catalyst (0.1 equiv to the theoretical resin loading). Double coupling of 1 h each is performed with both methodologies, and in both cases the hydroxyl group of the phenyllactic acid is esterified quantitatively as determined by Fmoc release UV assay. Two identical samples of the 3-*tert*-butyldisulfanyl-2-hydroxypropionamide PEGA resin obtained after the $KNO_2$ treatment are prepared. Then, one sample is functionalized with Fmoc-Phe using DCC via symmetrical anhydride while on the other Fmoc-Phe is coupled with HBTU/DIEA activation, in both cases with DMAP as catalyst as described above. Fmoc release UV assay on both resins after double coupling gives practically identical values. Thus, a model peptide of sequence LYKAF is synthesized in parallel with both resins and cleaved using a mixture of 95% TFA, 2.5% $H_2O$, 2.5% TIS (v/v) for 1 h. Surprisingly, the resin esterified using the HBTU/DIEA coupling yields 18% more of desired recovered product than the resin functionalized using DCC via symmetrical anhydride.

Due to the similarity with the CAM linker,[11] we expected our carboxyester linker to be stable to 20% piperidine solution necessary to remove the Fmoc group. To test our hypothesis, a sample of our resin already functionalized with Fmoc-Phe is treated with a mixture of 20% piperidine in anhydrous NMP, and the substitution is determined by Fmoc release UV assay. Another sample of such resin is treated for 5 h with 20% piperidine in anhydrous NMP (equivalent to the piperidine solution exposure necessary to synthesize a peptide of 50 residues using standard Fmoc cycles). At the end of 5 h treatment with 20% piperidine solution, the resin is still strongly positive to ninhydrin test. Fmoc-glycine is then coupled to the resin via *N*-hydroxysuccinimide/carbodiimide-mediated activation without any basic catalyst to avoid potential re-attachment of the FmocGly to the hydroxyl moiety eventually released by the piperidine exposure. The substitution is finally determined by Fmoc release UV assay. The compared results show no significant difference in the Fmoc release UV assay between the two samples, thus proving the stability of the carboxyester linker under prolonged piperidine treatment.

In our strategy, the peptide chain is then elongated and cleaved from the resin support by standard Fmoc chemistry procedures. Typically, HPLC of a crude with UV detection at 214 nm shows, beside the desired product, approximately 10−15% of an impurity that corresponds by Ms values to the hydrolyzed ester. Since in a separate experiment peptide-$C^{\alpha}OOCH-(CH_2SStBut)$-$CONH_2$ have been found to be completely stable in a mixture of 95% TFA, 2.5% $H_2O$, 2.5% TIS for over 3 h at r.t., we concluded that the side product is generated by a side reaction of the $KNO_2$ with the cysteinyl-Rink linker during the preparation of the resin. The desired peptide fragments are easily purified and stored for prolonged time without degradation or hydrolysis. Only under ligation conditions at neutral pH and in the presence of mild reducing reagents such as phosphines or appropriate nucleophiles such as thiophenol, is the free active thiol slowly liberated and the O to S acyl shift taking place. The newly formed thioester intermediate is then immediately captured by the excess of the N-terminal cysteine fragment present in solution.

Once the transthioesterification reaction with the C-terminal fragment has occurred, the "classical" S to N acyl shift takes place resulting in an amide bond formation that drives the whole process to completion.

Another important feature in this strategy is the protection of the reactive mercaptan through a disulfide bond. Such a scheme generally improves the stability of the carboxyester and avoids the formation of premature O to S acyl shift, thus simplifying purification. A further advantage of the S-tButhio protection is represented by the improved stability to nucleophiles compared to other S-alkyl or S-aryl moieties: indeed its use in combination with thiophenol allows a slow release of the free active thiol maintaining at each reaction time an artificial excess of the N-terminal cysteine fragment versus the newly rearranged thioester favoring kinetically the product of ligation.

Model peptides of sequence LYRAX-$C^{\alpha}OOCH-(CH_2SStBut)$-$CONH_2$ where X = Gly, Phe, and Lys were synthesized by standard Fmoc SPPS. When ligated at pH 7 in an equimolar ratio with a model fragment CYAKYAKL, the only side product detected was the hydrolyzed ester LYRAX-$C^{\alpha}OOH$ in a ratio between 10 and 20% to the ligated product as measured by HPLC with UV detection at 214 nm. As the control we reacted LYRAG-$C^{\alpha}OOCH_2$-$CONH_2$ with the fragment CYAKYAKL under identical conditions with no reaction occurring. To minimize the amount of hydrolyzed carboxyester, the reaction was carried out at pH 6.5 in the presence of 2% thiophenol (v/v) and a 1.5-fold excess of CYAKYAKL peptide. Under such conditions, LYRAF-$C^{\alpha}OOCH-(CH_2SStBut)$-$CONH_2$ (**2**) at a concentration of 2.5 mM was reacted with the model fragment CYAKYAKL (**1**) giving the ligated product LYRAFCYAKYAKL (**3**) and LYRAF-$C^{\alpha}OOH$ (**4**) as the side product in a ratio of 12.6% to the ligated product (Figure 2b). Importantly, the ligation product (**3**) was identical to the reference peptide having the same sequence and made by SPPS (Figure 2c). To improve the ligation yield, we also investigated the use of a single isomer carboxyester peptide. Thus, on a model peptide LYKAF-$C^{\alpha}OOCH-(CH_2SStBut)$-$CONH_2$ we have been able to separate the two diastereomers. Preliminary results however do not indicate dramatic changes in the amount of the hydrolyzed carboxyester: indeed, LYKAF-$C^{\alpha}OOCH-(CH_2SStBut)$-$CONH_2$ diastereomer A (more hydrophilic, early eluting peak in HPLC on C18

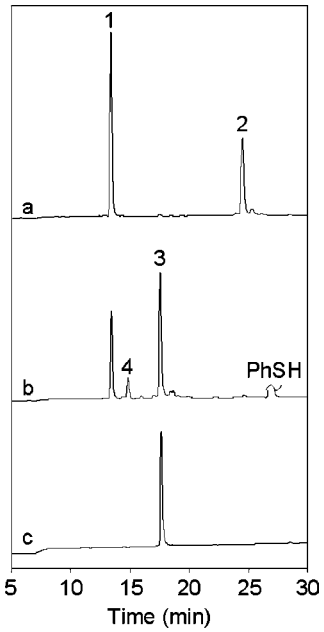



**Figure 2.** HPLC of ligation between model peptides LYRAF-C$^{\alpha}$OOCH-($CH_2$SStBu)-$CONH_2$ (**2**) with CYAKYAKL(**1**) (a) $T$ = 0, 1, (b) $T$ = 12 h, and (c) LYRAFCYAKYAKL obtained by stepwise SPPS.

column) when ligated with the model fragment CYAKYAKL at pH 6.5 in the presence of 2% thiophenol resulted in 13.8% of LYKAF-C$^{\alpha}$OOH, while the diastereomer B (late eluting peak) under identical conditions gave 10.5%. Since the ratio of the two diastereomers A/B is approximately 65/35%, our results on ligation with single diastereomer are in line with the results obtained utilizing the mixture.

To validate our strategy, we synthesized the linear form of NNY-Rantes (1−68)[17] and the phosphorylated spermatid nuclear transition protein 1 SPT1 1-54 using our approach. Thus, the synthesis of NNY-Rantes (1-68) by ligating two large fragments (more than 30 residues each) demonstrates that our ligation strategy can be successfully applied to synthesis of small proteins. Indeed, NNY-Pro2-Lys33Rantes-C$^{\alpha}$OOCH-($CH_2$SStBut)-$CONH_2$ is ligated with Cys34-Ser68 Rantes at pH 6.5 in the presence of 2% thiophenol (v/v) giving full length NNY-Rantes 1-68 and NNY-Pro2-Lys33Rantes-C$^{\alpha}$OOH in 19.5% yield relative to the ligated product as sole side product. The amount of hydrolyzed material is measured by HPLC with UV detection at 214 nm. Our experiment also indicates that our methodology is compatible with additional free cysteines present in the sequence. Furthermore, a parallel synthesis of NNY-Rantes (1−68) with 1% tributylphosphine (fast disulfide reducing agent) in the reaction mixture resulted in 25% of hydrolyzed material (relative to the ligated product) demonstrating the utility of the slow release of the free active thiol from the S-tButhio protection. Finally, the synthesis of phosphorylated spermatid nuclear transition protein 1 SPT1 1-54 is an example of the utility of our methodology to prepare proteins with PTM via NCL.

In summary, we presented a novel method that significantly extends the practical utility of native chemical ligation. Our strategy is easy, general, and fully compatible with standard Fmoc chemistry, and no additional manipulation or modification of the resin is required after completion of the desired sequence.

Additional improvements to this strategy are currently under development. In particular, our focus is on the solution synthesis of the 3-*tert*-butyldisulfanyl-2-hydroxypropionamide template (possibly chirally pure) ready to be incorporated to different types of resins, which will allow us to avoid the oxidative step on the PEGA resin. Furthermore, to reduce the amount of hydrolyzed material, we are exploiting different ligation conditions and different groups at the C-1 position. Future work will also try to address the mechanism of the formation of the hydrolyzed ester.

**Supporting Information Available:** Experimental procedures for the synthesis of linear NNY-Rantes 1-68, synthesis of phosphorylated spermatid nuclear transition protein 1 SPT1 1-54, and preparation of Fmoc-AA-C$^{\alpha}$oxy-(2-*tert*-butyldisulfanyl-1-carboxyamide)ethyl ester starting from Rink amide PEGA resin. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0481028

(17) Sabbe, R.; Picchio, G. R.; Pastore, C.; Chaloin, O.; Hartley, O.; Offord, R. E.; Mosier, D. E. *J. Virol.* **2001**, *75*, 661−671.