Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron Letters
Tetrahedron Letters 48 (2007) 2105–2107

Peptide dithiodiethanol esters for in situ generation of thioesters for use in native ligation

A. Pernille Tofteng,[a,b] Knud J. Jensen[a] and Thomas Hoeg-Jensen[b,*]

[a]*Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark*
[b]*Novo Nordisk, Novo Alle 6B2.54, DK-2880 Bagsvaerd, Denmark*

Received 10 November 2006; revised 15 January 2007; accepted 24 January 2007
Available online 30 January 2007

Abstract—A new approach is described for the general Fmoc-based solid-phase synthesis of C-terminal peptide (thio)esters. One hydroxy group of 2,2-dithiodiethanol (used in large excess) was anchored on trityl resin, and the remaining hydroxy group was loaded with the first amino acid. Standard chain elongation and TFA-based peptide release yielded peptide C-terminal dithiodiethanol esters in good purities. Under standard conditions of native chemical ligation (excess thiol, neutral pH), the dithiodiethanol function is presumably reduced and rearranged (or equilibrated) to the thioester via a 5-membered intermediate. The resulting thioesters are shown to undergo native chemical ligation with N-terminal cysteine peptides. Notably, hydrolysis of the reduced ester is a major competing reaction, especially in the presence of 6 M guanidinium chloride, which is often required for solubilization of large peptide fragments.

Native chemical ligation and expressed protein ligation have considerably extended the synthetic accessibility of peptides and proteins.[1,2] These methods rely on reactions between C-terminal thioester peptides/proteins and N-terminal cysteine peptides/proteins, all of which may be accessed synthetically or recombinantly. Peptide thioesters can be prepared synthetically by Boc-based solid-phase methods, but the strong acidic conditions of the final deprotection during Boc-based chemistry (hydrogen fluoride, etc.) precludes the synthesis of peptides with acid-sensitive functions, such as glycosides and phosphates. Fmoc-chemistry on the other hand tolerates such modifications, but unfortunately, thioester linkers are not stable during the repetitive treatments with piperidine commonly used for Fmoc-group removal. Numerous alternative methods for Fmoc-based syntheses of peptide thioesters have appeared, but none of them appear to be generally applicable.[3–8] For example, the use of a safety-catch linker demands an alkylation step upon peptide assembly.[3] This step is difficult, particularly with sequences containing histidine, which can undergo alkylation.

Recently, two new methods have appeared,[9,10] where peptide esters incorporating dithio functions are synthesised via Fmoc-chemistry, and where the dithio function is reduced during the native ligation conditions of excess thiol at neutral pH. The esters with liberated thiol functions can subsequently rearrange to thioesters which can participate in native ligation. However, each reported peptide dithio ester method has its limitations. One method applies diazotisation of a resin-bound cysteine, which demands a water compatible resin.[9] Also, the diazotisation generates a secondary alcohol for loading of the first amino acid, a process which has proven difficult. The other method utilizes esterification of the peptide C-terminal after peptide assembly, and this in turn demands that all auxiliary peptide carboxylates and amines be protected.[10]

We report here, the use of 2,2-dithiodiethanol as a linker for solid-phase peptide synthesis on 2-chloro-trityl resin using standard Fmoc-conditions to generate C-terminal peptide (thio)esters requiring no extra treatments prior to utilization in native ligation. The use of parentheses in *(thio)ester* is meant to indicate the presumed equilibrium of a 1,2-ester-thiol and a 1,2-thioester-alcohol, which is established upon in situ reduction of 2,2-dithiodiethanol esters. A set of peptide ester disulfides was prepared using the described method and examples

Keywords: Peptide thioester; Native chemical ligation; Dithiodiethanol linker; Hydrolysis.
* Corresponding author. Tel.: +45 30 79 13 01; fax: +45 44 44 42 56; e-mail: tshj@novonordisk.com

0040-4039/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2007.01.137

of utilization of the peptide ester disulfides in native ligation are shown. Notably, partial ester hydrolysis could not be avoided, and hydrolysis was the dominant reaction in buffers containing 6 M guanidinium chloride, as sometimes required for solubilization of larger peptides.

The rearrangement of a thiol ester to a hydroxyl thioester should theoretically be favoured by the 1,2-configuration of the hydroxy and thiol functions, since this geometry will allow the formation of a five-membered cyclic intermediate. Accordingly, 2,2-dithiodiethanol (DTDE) seemed an appropriate linker. DTDE was loaded onto 2-chloro-trityl chloride polystyrene resin (Scheme 1), utilizing a ten-fold excess of the linker in order to limit cross-linking of trityl sites. The first amino acids, Fmoc–Ala or Fmoc–Phe, were loaded on the DTDE linker by the standard amino acid anhydride method using DIC and DMAP, repeated twice. The standard UV/vis method was used for estimating the loading of the first amino acid, which was 0.12 mmol/g.[11] The amino acid loading was thus significantly lower than the starting resin substitution (0.72 mol/g), so partial DTDE cross-linking of the trityl resin may have occurred. However, the loading was still high enough to allow efficient peptide synthesis.

It was suspected that the dithio linker function would be somewhat labile to nucleophiles such as the piperidine used for repeated Fmoc-removal. Accordingly, the resin loading was monitored upon exposure to 20% piperidine in NMP for 10 min, 1 h and 3 h. The losses of loaded amino acids were 2%, 17% and 50% for Fmoc–Ala, and 1%, 15% and 32% for Fmoc–Phe, after 10 min, 1 and 3 h, respectively. Since 3 h of exposure to 20% piperidine equals the synthesis of a peptide with 30 residues using standard deprotections of 2×5 min per residue, the linker lability to piperidine is not disastrous even for long sequences. Disulfide-based linkers have previously been used for synthesis of oligonucleotides,[12,13] where ammonia is used repetitively for deprotections. Disulfide linker stability towards ammonia has been studied,[12] and the results were in line with our observations.

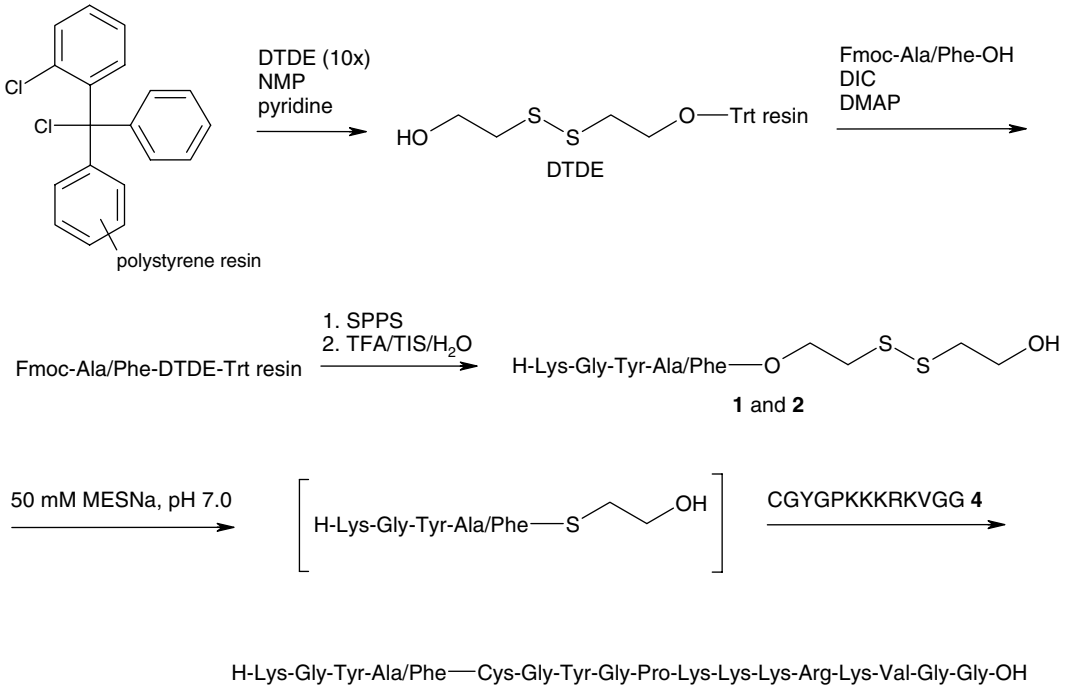
A set of peptide ester disulfides was prepared on the DTDE linker via standard Fmoc-based synthesis (alternating couplings of amino acids with DIC/HOBt in NMP and deprotections using 20% piperidine/NMP). The final peptide ester disulfides were cleaved from the resin using TFA/TIS/water 95:2.5:2.5, and the peptides were characterized by LCMS. A typical LCMS chromatogram of crude peptide DTDE ester **2** is shown in Figure 1. The peptides below were prepared by the described method and purified by RP-HPLC in water/acetonitrile/0.1% TFA. Yields are given in parentheses.

H-Lys-Gly-Tyr-Ala-DTDE (30%) **1**

H-Lys-Gly-Tyr-Phe-DTDE (25%) **2**

H-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-DTDE (45%) **3**

Native ligations of peptide esters **1** and **2** (2 mM) with the peptide H-Cys-Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly-OH (**4**, 1 mM) were studied on a 0.5 μmol scale using 0.1 M phosphate buffer with 50 mM MESNa, pH 7.0. Figure 2 shows the LCMS chromatogram of the reaction mixture from **2** and **4** after 20 h, with the individual components assigned. The desired product was isolated in 29% yield upon purifica-



Scheme 1.

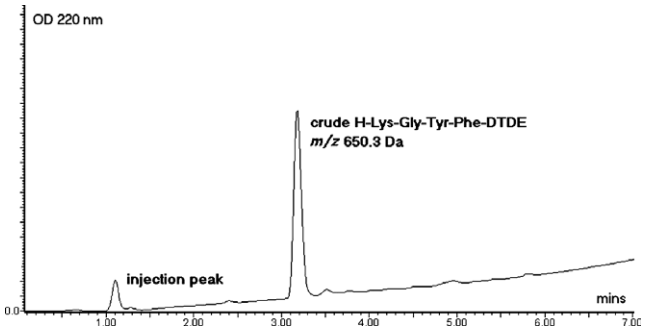


Figure 1. LCMS of crude H-Lys-Gly-Tyr-Phe-DTDE **2**.

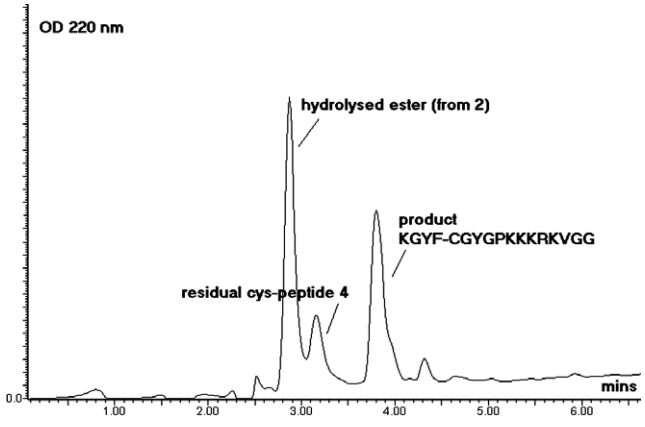


Figure 2. LCMS of the reaction mixture from ligation of peptides **2** and **4**, 20 h.

tion by RP-HPLC in water/acetonitrile/0.1% TFA. As is evident from Figure 2, ester hydrolysis was a significant competing reaction. Notably, peptides **1** and **2** could be dissolved in buffer without the assistance of chaotropic agents, but this is not the case for all peptides. Accordingly, ligation of peptide esters **1–3** were attempted in the presence of 6 M guanidinium chloride, 0.1 M phosphate buffer and 50 mM MESNa, pH 7.0. Unfortunately, only traces of ligation products could be detected in the reaction mixtures, and hydrolysed esters were the only major products under these conditions. It seems reasonable that the chaotropic effect of guanidinium chloride will induce a more solvent exposed conformation of the (thio)ester peptides, and thus increase the risk of hydrolysis. Attempts to optimize the conditions using a pH in the range 6–8 and other auxiliary thiols[14] did not improve the results.

In conclusion, DTDE has been shown to be a promising linker for the synthesis of peptide thioester precursors. Peptide DTDE esters can be prepared by standard Fmoc-based chain elongation and TFA-based deprotection, providing peptide DTDE esters in good yields and high purities. Under standard native ligation conditions, the DTDE esters are reduced and presumably rearranged to thioesters, which can undergo native ligation reactions. However, ester hydrolysis is a competing reaction and hence conditions with high concentrations of guanidinium chloride should be avoided.

References and notes

1. Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776–779.
2. Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705–6710.
3. Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369–11374.
4. Brask, J.; Albericio, F.; Jensen, K. J. *Org. Lett.* **2003**, *5*, 2951–2953.
5. Eggelkraut-Gottanka, R.; Klose, A.; Beck-Sickinger, A. G.; Beyermann, M. *Tetrahedron Lett.* **2003**, *44*, 3551–3554.
6. Camarero, J. A.; Hackel, B. J.; de Toreo, J. J.; Mitchell, A. R. *J. Org. Chem.* **2004**, *69*, 4145–4151.
7. Ollivier, N.; Behr, J. B.; El-Mahdi, O.; Blanpain, A.; Melnyk, O. *Org. Lett.* **2005**, *7*, 2647–2650.
8. Lu, Y. A.; Tam, J. P. *Org. Lett.* **2005**, *7*, 5003–5006.
9. Botti, P.; Villain, M.; Manganiello, S.; Gaertner, H. *Org. Lett.* **2004**, *6*, 4861–4864.
10. Warren, J. D.; Miller, J. S.; Keding, S. J.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 6576–6578.
11. Green, J.; Bradley, K. *Tetrahedron* **1993**, *49*, 4141–4146.
12. Salo, H.; Guzaev, A.; Lonnberg, H. *Bioconjugate Chem.* **1998**, *9*, 365–371.
13. Lack, O.; Zbinden, H.; Woggon, W. D. *Helv. Chim. Acta* **2002**, *85*, 495–501.
14. Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006**, *128*, 6640–6646.