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# Peptide $^{\alpha}$thioester formation using standard Fmoc-chemistry

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**Abstract**—A highly efficient and simple Fmoc-based preparation of peptide $^{\alpha}$thioesters is presented. After Fmoc/*t*-butyl solid-phase synthesis on 2-chlorotrityl resin the C-terminal carboxylic group of the protected peptide is directly converted to the corresponding thioester. The method leads to very high yields, shows a low level of epimerization and can be easily applied also for the preparation of long peptide $^{\alpha}$thioesters as demonstrated for the 41 amino acid N-terminal fragment of pro-neuropeptide Y (proNPY 1–40). 

C-terminal peptide thioesters can be used in many approaches[1] as starting compounds for the chemical synthesis of proteins particularly since the development of the native chemical ligation method (NCL, Fig. 1).[2,3] More than 300 biologically active proteins from numerous different families have been successfully synthesized by NCL.[4] Expanding the applicability of the chemical ligation via thioesters and N-terminally cysteine-bearing peptides requires effective preparation methods for its key intermediates: peptide thioesters. Whereas the intein technology[5,6] enables the molecular biological access to protein thioesters, peptide thioesters ($\leq 50$ amino acids) can be better obtained by chemical synthesis. This further provides the advantages of easily achievable substitutions at any position of the peptide sequence (e.g. by non canonical amino acids) or site-specific modifications of the peptide molecule (e.g. by fluorescent labels or biophysical probes). However, synthetic difficulties still restrict the chemical preparation of peptide $^{\alpha}$thioesters. Up to now most peptide thioesters are prepared by the so called Boc-strategy of solid-phase synthesis (Boc = *t*-butyloxycarbonyl). Disadvantages include strong acid treatment and hazardous operation conditions, which lead frequently to undesired side-reactions.[7–9] In contrast, Fmoc-strategy (Fmoc = 9-fluorenylmethyloxycarbonyl) implicates milder conditions and can be automated in parallel synthesis, yet it is limited by the susceptibility of thioester linkages to strong nucleophiles such as piperidine that is used for the removal of Fmoc groups. Several strategies for solving this problem have been reported. Specific cocktails for the removal of Fmoc groups keeping the thioester intact have been applied.[10–12,8] Within the backbone amide linker (BAL) strategy, an amino acid thioester is coupled to a fully protected, C-terminally free peptide anchored to a solid support through its backbone nitrogen. The final cleavage from the resin releases the peptide thioester.[13] More general seems to be a modification of Kenner's sulfonamide 'safety catch' linker. After synthesis the sulfonamide linker is activated by alkylation followed by cleavage of the peptide from the resin with a thiol nucleophile.[14] Without the need of a special linker a method using alkylaluminum thiolate for the cleavage of peptides from 4-hydroxymethylbenzoic acid (HMBA) and 4-hydroxymethyl-phenylacetamidomethyl polystyrene (Pam) resins yields peptide thioesters.[15,16] All these methods either require special agents/linkers or are reported to cause undesirable side-reactions. A fast, efficient and simple method for the preparation of peptide thioesters by Fmoc-based solid-phase chemistry remained to be established.

Such an attractive route results from the Fmoc-based synthesis of protected peptide acids on the commercially available Cl-trityl-resin followed by a direct conversion of the C-terminal carboxylic group to the corresponding thioesters. The peptide thioester is sufficiently stable towards acidic conditions, which allows the deprotection in TFA and even the following chromatographic purification in acetonitrile/water systems.

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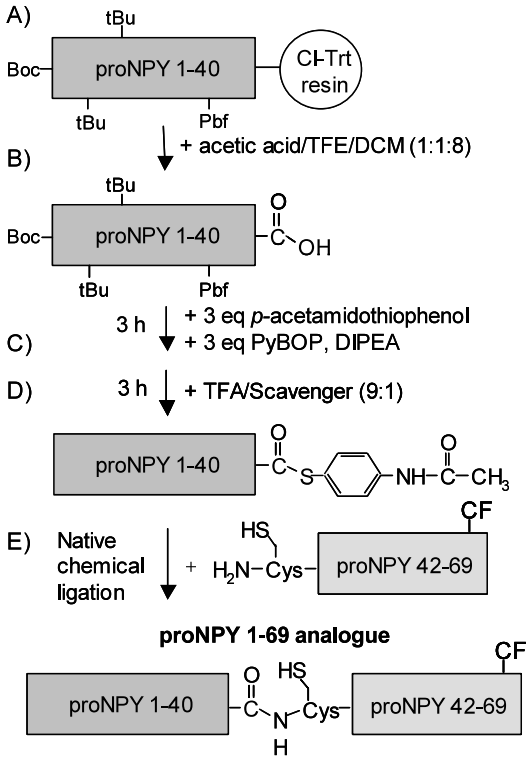


**Figure 1.** (A–D) $^{\alpha}$Thioester formation of proNPY 1–40: (A) Automated SPPS on Cl-Trt resin using Fmoc/tBu strategy; (B) fully protected cleavage from resin; (C) reaction with thiol; (D) deprotection of the peptide thioester; (E) NCL yields the full-length, carboxyfluorescein (CF) labeled proNPY analogue ([C$^{41}$, K$^{68}$(CF)]proNPY 1–69).

So far, application of this approach was complicated by the use of either a high excess of the activating agent (diisopropylcarbodiimide (DIPCDI, 20 equiv.)),[17] which requires an extra purification step before the deprotection, or the level of epimerization.[18] We have investigated conditions for the $^{\alpha}$thioester formation using a very low or no excess of activating agent. Different activating agents (benzotriazol-1-yl-*N*-oxy-tris-(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), benzo-triazol-1-yl-*N*-oxy-tris-(dimethyl-amino)phosphonium hexafluorophosphate (BOP), DIPCDI, and *N*-[(1*H*-benzotriazol-1-yl)-(dimethyl-amino)methylene]-*N*-methyl-methan-aminium tetra-fluoroborate *N*-oxide (TBTU)) and thiols (thiophenol, *p*-acetamidothiophenol, 3-mercapto-propionic acid ethyl ester) have been tested with respect to the rate of thioester formation and epimerization.

Initial studies were performed with Z-Gly-Ala-OH as a model substance. For analyzing different activating agents, the corresponding agent (0.01 mmol) was added to Z-Gly-Ala-OH (0.01 mmol) and *p*-acetamidothio-phenol (0.01 mmol) dissolved in 0.5 ml of dichloromethane (DCM). In the case of TBTU, BOP or PyBOP reactions were started by the addition of *N*,*N*-diisopropylethylamine (DIEA) (0.01 mmol). Progress of the reaction was monitored by analytical RP-HPLC. The product peaks were collected and identified with MALDI-MS. As indicated in Table 1, activation by phosphonium salts and carbodiimide, with or without *N*-hydroxybenzotriazol (HOBt), resulted in a fast thioester formation (more than 50% after 5 min) whereas the highest rate was achieved by PyBOP-activation. After 16 h (over night) all reactions have been run to completion with PyBOP or DIPCDI/HOBt, respectively. In contrast, TBTU could hardly activate Z-Gly-Ala-OH, a fact which can be attributed to a fast reaction between TBTU and the thiol prior to the activation of Z-Gly-Ala-OH (data not shown).

To optimize the reaction, different thiols were investigated. Significant differences in the thioester formation rate were observed in the rank order *p*-acetamidothiophenol>thiophenol>3-mercapto-propionic acid ethyl ester (Table 2). Moreover, *p*-acetamidothiophenol is a solid, shows convenient handling properties and has a low toxicity profile which makes it to our favored choice.

An effective thioester method comprises a low degree of stereomutation during its formation and the ligation step beside a high formation rate. We, therefore, carried out epimerization studies for Z-Gly-Ala-thioester obtained by activation with PyBOP or DIPCDI/HOBt, respectively, in combination with *p*-acetamidothiophenol. The resulting Z-Gly-Ala-S-acetamidophenyl esters (0.005 mmol) were ligated to H-Cys-OEt×HCl (0.005 mmol) in 1 mL of 0.1 M tris(hydroxymethyl)aminomethane (TRIS) buffer (pH 7.7) containing 3 M urea and $\beta$-mercaptoethanol (2 vol%). After reaction for 30 min an aliquot of the reaction mixture was applied to analytical RP-HPLC. ($t_R$(LL): 19.7 min, $t_R$(DL): 20.3 min). According to the HPLC data, we observed $\leq$3.5% stereomutation for PyBOP-activation and $\leq$1% for DIPCDI/HOBt-activation. The Z-Gly-Ala-OH used for the studies was optically pure (>99.9%) as checked by a control experiment, coupling Z-Gly-Ala-OH with DIPCDI/HOBt to H-Nle-OMe ($t_R$(LL): 23.00 min, $t_R$(DL): 23.3 min).

**Table 1.** Formation of Z-Gly-Ala-acetamidophenyl thioester using different activation methods

| Activating agent | Z-Gly-Ala-thioester, after 5 min (%) | Z-Gly-Ala-thioester, after 3 days (%) |
|---|---|---|
| PyBOP | 72 | 96 |
| BOP | 57 | 90 |
| TBTU | 3.5 | 13 |
| DIPCDI/HOBt | 56 | 99 |
| DIPCDI | 60 | 88 |

**Table 2.** Formation of Z-Gly-Ala-thioester using PyBOP and different thiols

| Thiol | Yield of Z-Gly-Ala-thioester after 5 min (%) |
|---|---|
| *p*-Acetamidothiophenol | 72 |
| Thiophenol | 50 |
| 3-Mercaptopropionic acid ethyl ester | 41 |

In order to apply the method to longer peptides, we have chosen the 21 amino acid (aa) segment of urocortin, acetyl-DDPPLSIDLTFHLLRTLLEIA-OH, and the 41 aa N-terminal fragment of pro-neuropeptide Y (proNPY 1–40),YPSKPDNPGEDAPAEDLARYY-SALRHYINLITRQRYGKRS-OH (Fig. 1).

Both peptide fragments were synthesized by Fmoc/*t*-Butyl solid-phase strategy with an automated peptide synthesizer on 2-chlorotrityl (Cl-Trt) resin which was preloaded with the first C-terminal amino acid. The protected peptides were cleaved from the resin with acetic acid/trifluorethanol/DCM (1:1:8 v/v/v) for 1–2 hours at room temperature. To remove acetic acid, the cleavage solution was in case of the 21-mer peptide diluted with *n*-hexane, evaporated, redissolved in dioxane and lyophilized. For proNPY 1–40, extraction of acetic acid with half-saturated $NaHCO_3$-solution proved to be superior to *n*-hexane; the DCM-phase was evaporated and the residue weighed for further reaction with thiol. A sample of each crude product was deprotected (21-mer peptide: TFA/phenol/triisopropylsilane/$H_2O$, 87.5/5/2.5/5; proNPY 1–40: TFA/thioanisole/*p*-cresol, 90/5/5, 3 h, rt) and analyzed by RP-HPLC and mass spectrometry revealing a content for the 21-mer peptide of 54% (ESI-MS: $M_{calc.}$: 2434.8; $M_{found}$: 2435.0) and $\geq$70% for proNPY 1–40 (MALDI-MS: $[M+H]_{calc.}$: 4684.2, $[M+H]_{found.}$: 4684.2).

The thioester of the 21-mer peptide was formed by treating the crude protected peptide (380 mg, 0.11 mmol) with DIPCDI (1.5 equiv.) and *p*-acetamidothiophenol (15 equiv.) in DCM (20 mL) over night. The solvent was evaporated and the residue deprotected as described above. After precipitation with ice-cold diethyl ether the deprotected peptide thioester was purified by preparative RP-HPLC in a $H_2O$–acetonitrile (0.1% TFA) system. The purified peptide thioester ($[M+H]_{calc.}$: 2582.4, $[M+H]_{found.}$: 2583.4) was obtained in a considerable yield of 47% (76 mg) based on the peptide content of the crude product.

The optimum for the thioester formation of proNPY 1–40 was obtained by adding PyBOP (3 equiv.), *p*-acetamidothiophenol (3 equiv.) and DIEA (3 equiv.) to the crude protected peptide (13 mg, 0.0016 mmol) dissolved in DCM (20 mL). After 1.5–3 h reaction at rt the solvent was evaporated and the residue deprotected as described above. The peptide thioester was characterized by analytical RP-HPLC (Fig. 2) and MALDI-MS ($[M+H]_{calc.}$:4833.4, $[M+H]_{found}$: 4834.2). The thioester was formed with a yield of >95%. With respect to the peptide content in the crude product, a final yield of 70% (5.4 mg) of the thioester product was achieved. The optical purity of the peptide thioesters was analyzed after hydrolysis in 6 N $D_2O$/DCl by GC–MS. In case of the acetyl-urocortin(1–21) thioester a D-alanine content of 0.2% was determined and for the C-terminal amino acid of the proNPY 1–40 a content of 1.39% was found (D-serine).

Native chemical ligation reactions demonstrated the suitability of the generated peptide thioesters. The 21 aa

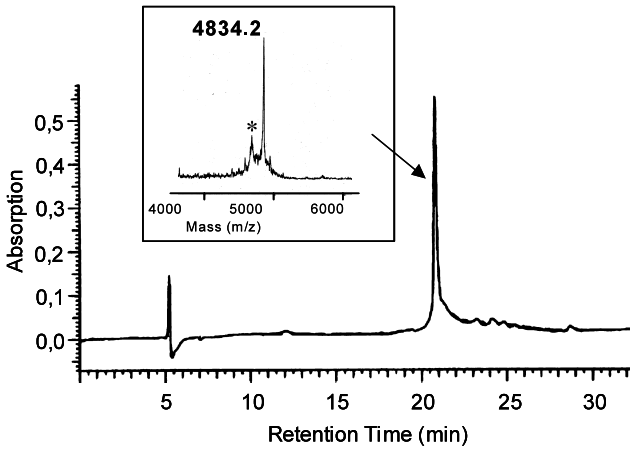


**Figure 2.** RP-HPLC (lower panel; gradient from 10 to 70% ACN in water over 30 min at a flow rate of 0.6 ml/min) and MALDI-MS (upper panel) profiles of the crude proNPY 1–40 acetamidophenyl thioester, *corresponds to the hydrolyzed thioester due to MALDI measurements.

urocortin segment thioester (0.5 mg, 0.18 mmol) dissolved in 2 mL NMP was mixed with 8 ml of a 0.2 M solution of H-Cys-OEt×HCl in 0.1 M $NaHCO_3$ buffer (pH 8.5) containing 3 M urea. The progress of the reaction was monitored by RP-HPLC. The reaction was nearly completed (>90%) after 1 h. ESI-MS confirmed the correct mass of the ligation product ($[M+H]_{calc.}$: 2565.4, $[M+H]_{found.}$: 2565.1). This model reaction shows the applicability of the acetyl-urocortin segment thioester for couplings to peptide libraries in connection with structure–activity relationship studies of corticotrophin-releasing-factor (CRF) receptor ligands.[19]

Ligation of the thioester fragment of proNPY 1–40 and the corresponding N-terminal cysteine fragment, labeled with carboxyfluorescein (CF), [$C^{41}$, K68(CF)] proNPY 41–69, was performed in γ-morpholinopropanesulfonate (MOPS) buffer (0.1 M), pH 7.5, containing 6 M guanidine×HCl. After dissolving of both reactants (1 mM) thiophenol (2%) and benzyl mercaptan (2%) were added. The ligation mixture was stirred at room temperature for 8 h and monitored by analytical RP-HPLC. The ligation product was purified by semi-preparative RP-HPLC and analyzed by MALDI-MS ($[M+H]_{calc.}$: 8425.4, $[M+H]_{found}$: 8425.4).

In conclusion, the preparation method presented here provides a highly efficient, fast and low-cost way for the generation of peptide thioesters by Fmoc-based solid phase strategy. The method can be applied also to the thioester synthesis of long peptides as demonstrated by the synthesis of the 41 aa proNPY 1–40. By using a very low excess of activating reagents previous limitations of the method could be overcome. We could show for the first time that particularly phosphonium salt based reagents in the presence of thiol are very suitable for an excellent thioester formation in high yields

(>95%) while keeping epimerization on a very low level (<1.4%). Because of its simplicity and the usage of only commercially available resin and reagents, the method shows a wide applicability for the synthesis of the thioester intermediate of NCL.

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