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Synthesis and Use of a Pseudo-cysteine for Native Chemical Ligation

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Abstract: The process of native chemical ligation (NCL) is well described in the literature. An *N*-terminal cysteine-containing peptide reacts with a *C*-terminal thioester-containing peptide to yield a native amide bond after transesterification and acyl transfer. An *N*-terminal cysteine is required as both the *N*-terminal amino function and the sidechain thiol participate in the ligation reaction. In certain circumstances it is desirable, or even imperative, that the *N*-terminal region of a peptidic reaction partner remain unmodified, for instance for the retention of biological activity after ligation. This work discusses the synthesis of a pseudo-*N*-terminal cysteine building block for incorporation into peptides using standard methods of solid phase synthesis. Upon deprotection, this building block affords a *de facto N*-terminal cysteine positioned on an amino acid sidechain, which is capable of undergoing native chemical ligation with a thioester. The syntheses of several peptides and structures containing this motif are detailed, their reactions discussed, and further applications of this technology proposed.

Keywords: native chemical ligation; pseudo-cysteine; reactive scaffold; intein

INTRODUCTION

It is known that it is possible to ligate two peptidic or non-peptidic compounds together by a process known as native chemical ligation (Figure 1) [1]. The basic requirements for this coupling are the presence on participant **A** of a thioester moiety such as a benzyl or ethyl thioester, and the presence on participant **B** of an aminothiol. The coupling reaction commences when the thio-nucleophile attacks the thioester in a reversible fashion with loss of the thioalkyl group. The thioester so formed then undergoes an intramolecular $S \rightarrow N$ acyl shift to produce irreversibly the ligated amide product **A-B** with a native cysteine at the junction (Figure 1). If it is possible to unmask a further *N*-terminal cysteine at the *N*-terminal of newly formed ligated product **A-B** this process can be repeated [2]. This unmasking can be achieved by enzymatic means or by classical protecting group chemistry [3]. A further native chemical ligation coupling with a thioester-containing moiety **E** would therefore yield the product **E-A-B**. These methods have been used to generate a variety of proteins. It is also known that cyclic peptides/proteins can be produced by incorporating both the thioester and amino terminal cysteine moieties within the same molecule [4]. Furthermore, it is possible to perform the ligations described above without removing all of the reaction participants from a solid phase [2,5,6].

A method for incorporating a thioester moiety into a reaction participant where one does not otherwise exist has been described [7]. This method

Abbreviations: Amino acid and other peptide-related abbreviations are incorporated with reference to guidelines published in *J. Peptide. Sci.* 1999; **5**: 465–471. Amino acids used in this work are of the L-configuration; NCL, native chemical ligation; MSWP, myristoyl electrostatic switch peptide; EDT, ethanedithiol; TIPS, tri-isopropylsilane; EGFP, enhanced green fluorescent protein; IPTG, isopropyl thiogalactoside; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MESNA, 2-mercaptoethylsulfonate; SGC, silica gel chromatography; TCEP, tris-2-carboxyethyl phosphine.

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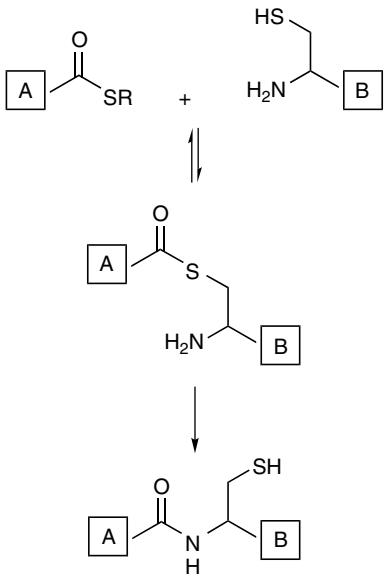
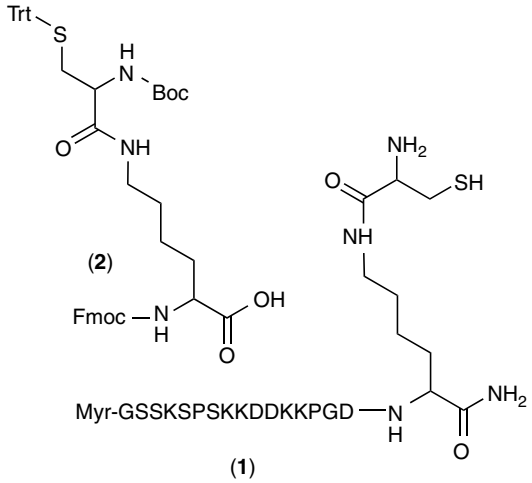


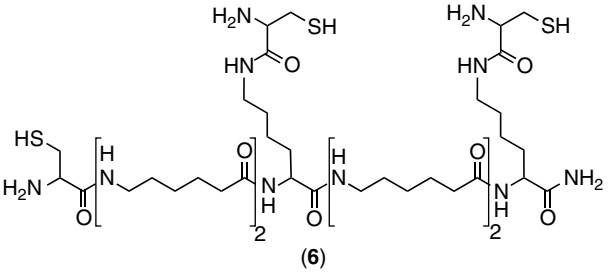
Figure 1 Native chemical ligation (NCL).

relies on the reaction of a primary amine with thiolane-2,5-dione and subsequent alkylation of the thioacid formed with an appropriate alkyl bromide. This method requires a single primary amine to be present otherwise multiple thioesters may be coupled together.

Under certain circumstances it is desirable to perform a native chemical ligation not involving an *N*-terminal cysteine. For instance in our work we needed to ligate a myristoyl electrostatic switch peptide (MSWP10 **(1)**) [8] to a therapeutic peptide by NCL. **(1)** Comprises a net basic 17-mer peptide with an *N*-terminal myristoylation essential for its biological activity and modified pharmacokinetics. The *N*-terminal of the therapeutic peptide was also required unmodified for biological activity. We needed, therefore, to generate a pseudo-*N*-terminal cysteine at the *C*-terminal of the peptide **(1)**, to allow NCL of the two peptides.

Certain amino acid monomer molecules are available with a protected functionality grafted onto an amino acid sidechain. For example, Fmoc-(Boc-amino-oxyacetyl)-βAla-OH [9] (Novabiochem, San Diego, CA) incorporates a protected oxyamine onto an amino acid sidechain that is capable, after deprotection, of undergoing chemoselective ligation to carbonyl containing moieties. Monomer **(2)** was synthesized, which incorporates a minimal 1-amino-2-thiol moiety in masked form onto the ε-amino group of Fmoc-Lys-OH. This derivative is easily incorporated into a solid phase Fmoc synthetic procedure, and yields, after cleavage and deprotection, a pseudo-*N*-terminal cysteine at the *C*-terminal of the peptide.





The theoretical uses of such a chemical motif are various. It may be incorporated at any point in a peptide chain. Indeed, multiple pseudo-cysteines are possible to generate a reactive scaffold for NCL. An exploration of some of these possibilities will be described.

MATERIALS AND METHODS

Synthesis of Protected Monomer (2)

Fmoc-Lys(Boc)-OH (5.14 g, 11.0 mmol) was deprotected in 50/50 DCM/TFA (~20 ml) over 30 min. The solvent was evaporated under reduced pressure to furnish Fmoc-Lys-OH trifluoroacetate. A solution of Boc-Cys(Trt)-OH (4.66 g, 10.0 mmol) and *N*-hydroxysuccinimide (1.15 g, 10.0 mmol) in 1,4-dioxane (20 ml) and DCC (2.06 g, 10.0 mmol) were

stirred overnight. Urea precipitate was filtered off and washed with DCM. Solvent was evaporated and the resulting oil dissolved in ethyl acetate, then washed with saturated sodium carbonate solution then water. After drying (Na_2SO_4), the solvent was removed under reduced pressure to yield Boc-Cys(Trt)-OSu. Fmoc-Lys-OH trifluoroacetate above was dissolved in DCM (20 ml) and Boc-Cys(Trt)-OSu above (dissolved in DMF (30 ml) and DCM (10 ml)) added. The solutions were mixed and DIPEA added to pH 7. After overnight stirring at ambient temperature, half the solvent was removed under reduced pressure, and ethyl acetate (200 ml) added. The mixture was washed with water, sodium bicarbonate and water. The organic layer was dried (Na_2SO_4), and evaporated to yield a cream-coloured foam. The product was purified using SGC using ethyl acetate/petroleum ether 40–60 to afford the product as a pale yellow solid (5.8 g, 71%); m/z (ESI^+) 814 $(M+H)^+$; ^1H NMR† ($CDCl_3$) δ: 1.40 (s, 9H, 3 × Boc C$\underline{H}_3$), 1.60–1.95 (m, 6H, Lys β-C$\underline{H}_2$ + Lys γ-C$\underline{H}_2$ + Lys δ-C$\underline{H}_2$), 2.45–2.65 (m, 2H) 3.15 (br s, 2H, Cys β-C$\underline{H}_2$), 3.75 (br s, 2H, Lys ε-C$\underline{H}_2$), 4.20–4.50 (5H, m, Fmoc C$\underline{H}$ + C$\underline{H}_2$, Lys α-C$\underline{H}$, Cys α-C$\underline{H}$), 5.00 (br, 1H, N$\underline{H}$), 5.75 (br, 1H, N$\underline{H}$), 6.15 (br, 1H, N$\underline{H}$), 7.20–7.75 (m, 23H, 8 × Fmoc C$\underline{H}$ + 15 × Trt C$\underline{H}$); ^{13}C NMR ($CDCl_3$) δ: 21.8, 28.2, 28.5, 31.3, 33.6, 38.6, 47.1, 53.6, 53.6, 67.0, 67.2, 80.6, 119.9, 125.2, 126.9, 127.1, 127.7, 128.0, 129.5, 141.2, 143.7, 143.8, 144.3, 155.6, 156.1, 170.8, 170.8; M.Pt. 114-116 °C; $[\alpha]_d^{25} + 7°$ (MeOH, c = 0.5).

Synthesis of Native Chemical Ligation Peptide MSWP10 (1)

Myristoyl-GSSKSPSKKDDKKPGDK-(ε-amino-cysteinyl)-NH_2 (**1**) was prepared by manual solid phase synthesis, using Fmoc synthesis on Rink amide resin (Advanced ChemTech, Sandy, UK). Coupling reactions were carried out using appropriately protected Fmoc amino acids (Advanced ChemTech) activated with HBTU and HOBt (Advanced ChemTech) aided with DIPEA, with ninhydrin monitoring after each extension. The first amino acid was installed as **(2)** and Fmoc protection removed with 20% piperidine. The remainder of the synthesis was carried out using appropriately protected Fmoc monomers. The *N*-terminal myristoyl function was incorporated using myristic acid and standard coupling conditions. Cleavage from the resin and deprotection of the sidechain protecting groups was accomplished with a mixture of TFA/EDT/TIPS/water (90/5/2.5/2.5 v/v/v/v, 5 ml) added to the reaction vessel and shaken for 1.5 h. The mixture was blown into a 50 ml centrifuge tube with nitrogen, and DCM (<5 ml) used to wash through any remaining compound. The mixture was then shaken for a further 1.5 h added to ice-cold diethyl ether (35 ml), the tube vortexed then centrifuged at 3500 rpm for 5 min. The ether was decanted. The resultant solid was washed (vortexed/centrifuged/decanted) with three more quantities of ice-cold diethyl ether (10 ml). The crude peptide was desalted by gel filtration (Sephadex G10, 0.1% TFA in water) before purification by preparative C18 Vydac HPLC using 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid/90% acetonitrile as gradient components. After lyophilization, the peptide was a white amorphous powder, soluble to at least 10 mg/ml in both DMSO and water. MS (FAB^+) 2101 $(M+H)^+$.

Expression of Recombinant EGFP in the Intein System

The gene for enhanced green fluorescent protein was expressed under the control of the T7*lac* promoter as a *C*-terminal fusion with the VMA1 gene for the intein from *Saccharomyces cerevisiae* and the gene for the chitin binding domain from *Bacillus circulans* [10]. The expression plasmid was a kind gift from Dr Gerald Böhm, Martin-Luther-Universität Halle (Saale).

Upon transformation into the bacterial strain Hams113 (Personal Communication, Dr Michael Steward, Adprotech Ltd) an overnight starter culture of 25 ml LB medium containing 100 μg/ml ampicillin was inoculated with a single colony and, on the next morning, diluted 1 : 100 into 2 l of LB medium containing 100 μg/ml ampicillin. The culture was fermented at 37 °C until an optical density at 600 nm of 0.5 was achieved, at which point the temperature was lowered to 20 °C and expression of the gene induced by the addition of 1 mM IPTG after 30 min. Cells were harvested 21 h post induction by low-speed centrifugation and stored in aliquots at −40 °C.

Affinity Purification of EGFP

To lyse cells, an aliquot of bacterial paste corresponding to 333 ml of fermentation broth was

† NMR spectra were recorded on a Bruker AMX2-400 NMR spectrometer at the University of Sheffield by Sue Bradshaw and Dr Brian Taylor. Mass spectrometry was carried out on a Micromass LCT in electrospray mode.

resuspended in 40 ml of ice-cold buffer 100 (20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM EDTA) and homogenized by three passages through an Emulsiflex C5 homogenizer at a pressure of 12 kpsi. The soluble fraction was obtained by centrifugation (20 000 × g, 30 min, 4 °C) and immediately passed over a column containing 15 ml chitin beads (New England Biolabs). The column was washed with four column volumes of buffer 100, eight column volumes of buffer 2000 (20 mM HEPES pH 8.0, 2000 mM NaCl, 1 mM EDTA), four column volumes of buffer 100, and finally with three column volumes of cleavage buffer 1 (buffer 100 containing 50 mM cysteine). After incubation at 6 °C for 20 h, EGFP was eluted from the column using buffer 100 as the elution buffer.

Linkage of (1) to EGFP by Native Chemical Ligation

EGFP was purified as described above, but cleavage buffer 2 (buffer 100 containing 50 mM sodium 2-mercaptoethylsulfonate (MESNA) and 50 μM **(1)**) was used in the overnight incubation. The cleavage resulted in almost quantitative derivatization of EGFP with a single 2 kDa peptide as evidenced by SDS PAGE.

Synthesis of Linker Molecule (3)

Standard Fmoc solid phase synthesis was utilised on Rink amide resin. Monomer **(2)** was coupled first followed by Fmoc-9-aminononanoic acid (Advanced ChemTech) followed by Boc-Cys(Trt)-OH. After cleavage from the resin and removal of all protecting groups using TFA/EDT/TIPS (90/5/5) the crude linker was ether precipitated and purified as described for **(1)**. m/z (ESI)$^+$ 507.2 $(M + H)^+$.

Formation of a 'Homodimer' (4) Using Simultaneous 'Double NCL'

A solution of **(3)** in water (37.5 mM, 21.3 μl) was added to a solution of TCEP (tris-2-carboxyethyl phosphine) (1 equiv) in HEPES buffer (pH 8.5, 40 mM, 265 μl). A solution of APT2501 (Myr-GSSKSPSKKKKKKPGDG-ethyl thioester — synthesized by PPR Ltd) (10 mg/ml, 140 μl, 10-fold excess) was then added. The reaction mixture was left at ambient temperature overnight and then analysed by HPLC. The doubly ligated product eluted slightly later than APT2501 and was found to be a 2/1 ligation product by MALDI MS, 4378 $(M + H)^+$. This product was purified by preparative HPLC and lyophilized.

Formation of 'Heterodimer' (5) using Sequential 'Double NCL'

A solution of **(3)** in water (37.5 mM, 21.3 μl) was added to a solution of TCEP (tris-2-carboxyethyl phosphine) (1 equiv) in HEPES buffer (pH 8.5, 40 mM, 265 μl). A solution of APT2501 (1 mg/ml, 140 μl) was then added. The reaction mixture was left at ambient temperature overnight and then analysed by HPLC. The ligated product eluted slightly later than APT2501 and was found to be a 1/1 ligation product by ESI$^+$, 2444 $(M + H)^+$. This product was purified by preparative HPLC and lyophilized. EGFP MESNA thioester lyophilizate was dissolved in HEPES buffer (pH 8.5 40 mM, 250 μl) and added to a solution of TCEP (tris-2-carboxyethyl phosphine) (1 equiv) in HEPES buffer (pH 8.5, 40 mM, 265 μl). A solution of the singly ligated product described above in the same buffer (25 fold molar excess) was then added and the mixture incubated overnight at ambient temperature. SDS PAGE showed clear and quantitative conversion of the protein thioester to a ligated product approximately 2 kDa higher in molecular weight.

Synthesis of Trimerization Scaffold (6)

Standard Fmoc solid phase synthesis was utilized on Rink amide resin. Monomer **(2)** was coupled first followed by Fmoc-6-aminocaproic acid (twice) followed by monomer **(2)**. Two further additions of Fmoc-6-aminocaproic acid and one of Boc-Cys(Trt)-OH completed the synthesis. After cleavage from the resin and removal of all protecting groups using TFA/EDT/TIPS (95/5/5) the crude linker was ether precipitated and purified by preparative C18-reverse phase HPLC. m/z (ESI)$^+$ 1035.4 $(M + H)^+$.

RESULTS

The 'pseudo-cysteine' Fmoc synthesis monomer **(2)** was successfully synthesized in three convergent steps from commercially available starting materials (Figure 2). Fmoc-Lys(Boc)-OH was deprotected at the ε-nitrogen by treatment with 1/1 dichloromethane/trifluoroacetic acid. Work-up consisted of evaporation and freeze-drying to yield Fmoc-Lys as its trifluoroacetate salt in essentially quantitative yield. Independently, Boc-Cys(Trt)-OH was treated with N-hydroxysuccinimide and DCC to yield the activated ester in excellent yield (both of

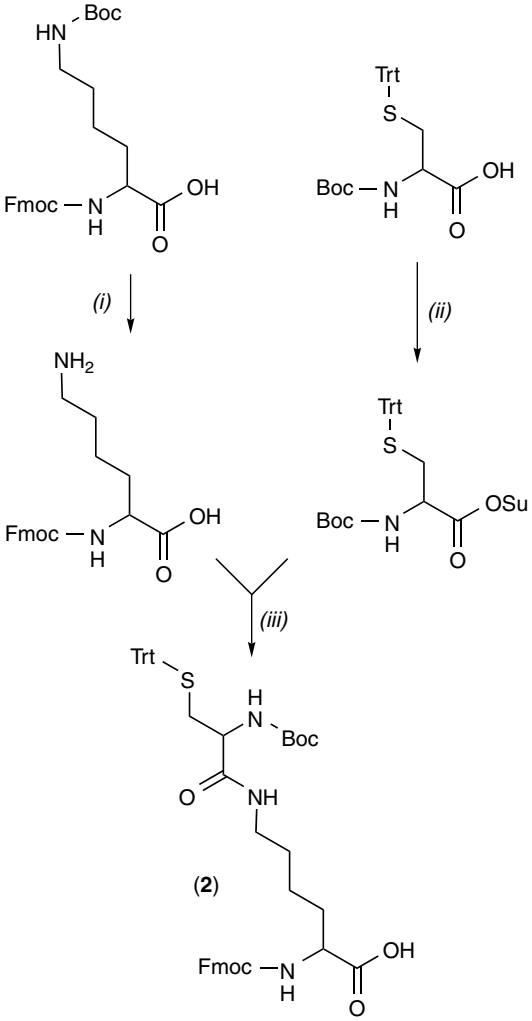


Figure 2 Synthesis of monomer **(2)**. (i) 1/1 DCM/TFA; (ii) *N*-hydroxysuccinimide/DCC/dioxane; (iii) DIPEA (pH 7).

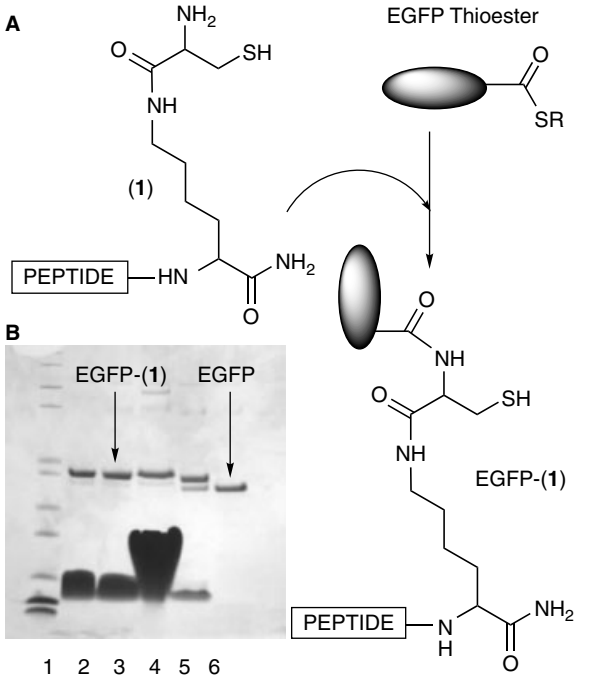


Figure 3 A. NCL reaction of EGFP thioester with **(1)**. B. SDS PAGE gel of conjugation: lane 1, mark 12 molecular weight markers (Invitrogen); lane 2, EGFP-MESNA thioester + 250 μM **(1)** non-reduced; lane 3, EGFP-MESNA thioester + 250 μM **(1)** reduced; lane 4, EGFP-MESNA thioester + 5 mM **(1)**; lane 5, EGFP-cleavage with 50 μM **(1)**; lane 6, EGFP-MESNA thioester. Invitrogen NuPAGE 4–12% 1.0 mM Bis-Tris gel run with MES SDS running buffer from 20x concentrate.

these materials are also available commercially, e.g. Advanced ChemTech). The two were reacted together in the presence of DIPEA to yield monomer **(2)** in good yield after work-up and purification by silica gel chromatography.

Monomer **(2)** was incorporated easily into standard Fmoc solid phase peptide synthesis protocols. In the case of MSWP10 **(1)**, the monomer was added to Rink amide resin at the first stage of synthesis. The Boc, Trt protected pseudo-cysteine remains unchanged during the rest of the synthesis and isolation. Purification was straightforward to yield **(1)** (Figure 3) which was isolated as a white lyophilizate. The availability of the thiol was confirmed by Ellman's assay (data not shown).

In order to demonstrate that **(1)** was capable of undergoing native chemical ligation *via* the pseudo-cysteine, it was incubated overnight with a MESNA thioester of EGFP (see experimental section for preparation of this reagent). SDS PAGE analysis of the protein before and after treatment with **(1)** showed a clear and quantitative increase in weight of ~2 kDa corresponding to the addition of MSWP10 (**1**) (Figure 3). Increasing concentrations of **(1)** in the mixture (lanes 2–4 of Figure 3B) did not result in any further derivatization of the protein indicating that the reaction was chemoselective. It was also possible, though not in the same quantitative fashion, to derivatize EGFP directly with **(1)** by inclusion of **(1)** in the cleavage buffer in place of MESNA (lane 5 of Figure 3B). The reduced yield compared to reaction with the MESNA thioester was probably due to steric factors. The existence of the native amide bond in the derivatized protein was confirmed by the observation that

the conjugate was impervious to reductive (2-mercaptoethanol) cleavage.

Using monomer **(2)** it was possible to generate a small molecule linker capable of performing NCL at both of its ends. Synthesis was once again a simple extension of standard Fmoc solid phase synthesis to incorporate a true *N*-terminal cysteine at one end of linker **(3)** and a pseudo-cysteine at the *C*-terminal (Figure 4).

Linker **(3)** was able to react in two modes. Firstly, it could be reacted with an excess of a single thioester (e.g. APT2501) to generate a pseudo-symmetrical dimer molecule **(4)** linked *via* a short peptide spacer (Figure 4). Alternatively, **(3)** could be reacted in excess with one thioester-containing molecule (APT2501), the product purified, then reacted with a second thioester- containing molecule (EGFP thioester) to yield a conjugate such as **(5)** (Figure 5).

The type of molecule exemplified by **(3)** can be extended in theory to incorporate multiple pseudo-cysteines to act as a scaffold for the addition of a commensurate number of identical molecules by NCL. This concept was demonstrated in a first order fashion by the synthesis of trimerization scaffold **(6)**. The synthesis was analogous to that of **(3)** but yielded a molecule capable of supporting three separate NCL reactions, subject obviously to steric constraints.

DISCUSSION

Native chemical ligation is an increasingly popular technique used in a number of disciplines. The main advantage of the technique is that it is chemoselective: only *N*-terminal cysteines can react with thioesters through to formation of the native amide bond, allowing ligation to proceed under aqueous conditions with no protecting groups required *per se*. This synthetic procedure has made a number of protein targets accessible that would otherwise have been prohibitively unwieldy using protected block synthesis. NCL is also useful for more general conjugation chemistry, and has the advantage that the product conjugate contains a biologically benign amide linkage. Other linking chemistries have their potential drawbacks: linking *via* other types of sulfur chemistries yields potentially immunogenic succinamide thioethers in the case of maleimide chemistry, reductively cleavable disulfides in the case of disulfide exchange, and unnatural thioethers in the case of thio/halide couplings. These chemistries in common with amine/activated ester couplings also suffer from potential limitations in terms of regioselectivity.

Despite all of the advantages of NCL, one possible drawback remained until now: the requirement for a cysteine at the *N*-terminal of one of the reaction partners. In this work it was demonstrated

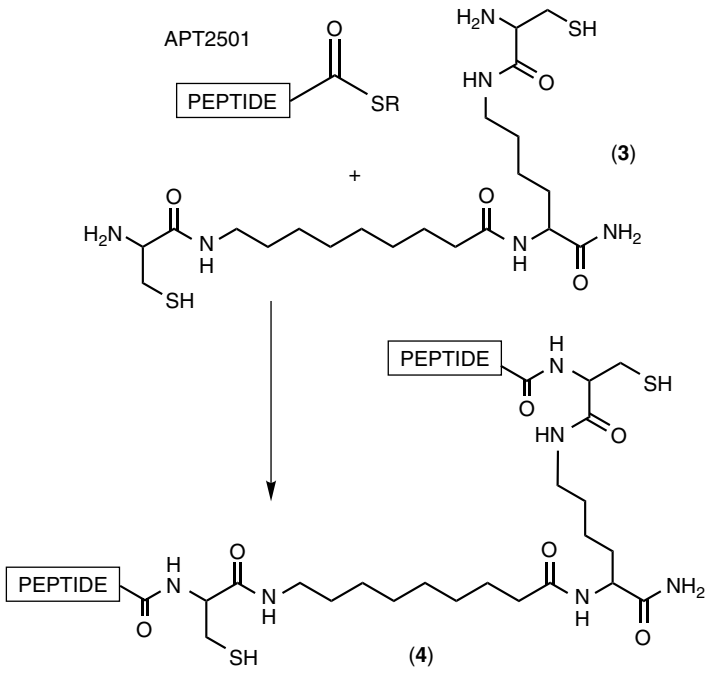


Figure 4 Synthesis of 'homodimer' **(4)** by simultaneous 'double-NCL'.

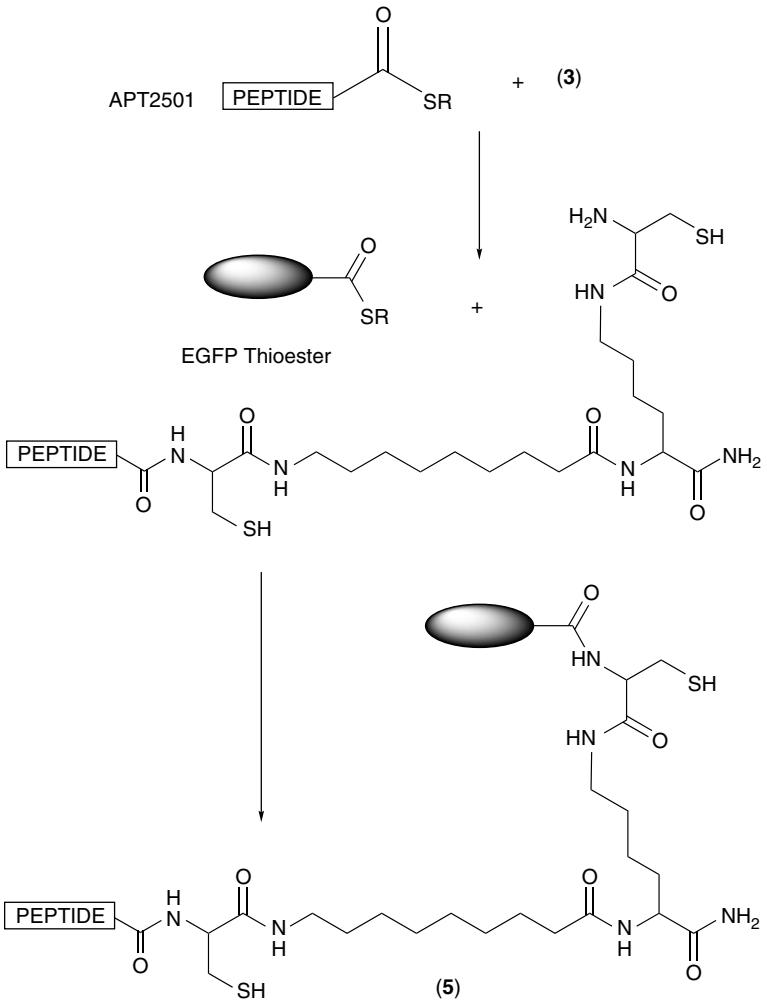


Figure 5 Conjugation of protein to peptide by sequential 'double NCL'.

that using a monomer **(2)** available in three convergent chemical steps, it is possible to install a pseudo-cysteine motif anywhere in a peptide synthesized by standard Fmoc synthesis. The monomer can be incorporated during a manual synthesis or during synthesis on an automated peptide synthesizer (demonstrated in our hands on an Advanced ChemTech ACT348ΩMPS multiple peptide synthesizer) as is required. We have demonstrated the ability of the pseudo-cysteine to undergo NCL by ligating **(1)** to a thioester form of enhanced green fluorescent protein, with stable ligation confirmed by gel shift analysis and MALDI mass spectrometry. This was achieved either by direct cleavage from the intein purification by **(1)** or *via* the intermediacy of a MESNA thioester.

In a logical extension of this concept it was also demonstrated that it is possible to use a single molecule containing more than one *N*-terminal cysteine to act as a linker between more than one thioester-containing species. In its simplest proof of concept form, it was shown that linker **(3)** can be reacted with an excess of a peptide *C*-terminal thioester and the dimerized product can be obtained by HPLC with the correct molecular mass. Furthermore, dimerized product was shown to be stable to reduction with TCEP or excess 2-mercaptoethanol, again confirming a true NCL reaction. Also, Ellman's assay of the product showed that the product contained free thiols indicating further that a true NCL reaction has occurred at each junction site and not a thioesterification. The utility of this linker was then extended to conjugate two different thioester-containing moieties. This type of conjugation required first the reaction of **(3)** with APT2501 as described previously, but in this

instance with **(3)** in excess. This ensured that all of the APT2501 was reacted to form a 1 : 1 ligation product with **(3)**, then incubated with EGFP MESNA thioester to perform the second NCL to protein. Once more it was demonstrated that the product **(5)** was a true product of successive NCL reactions, by showing that the clear 2 kDa gel shift on ligation of the peptide to protein remained even after subjecting the conjugate to reductive conditions. Due to the non-symmetrical nature of the linker **(3)** it should be pointed out that the product **(5)** of this reaction is a mixture of two isomeric forms, depending on which end of linker **(3)** reacted first with APT2501. Despite this it is clear that the two forms are functionally identical.

Further extensions of this methodology are clearly possible. We have shown that it is possible to incorporate multiple pseudo-cysteines into peptides using SPPS, which may prove of utility if multiple identical motifs are to be linked using NCL. These can be closer or further apart depending on the steric demands of the thioesters. Although it is possible using linker **(3)** to selectively link two different molecules together in a defined fashion, it is difficult to envisage being able to control more than this number where the number of pseudo-cysteines is greater than two. A possible method to accomplish this would be to use orthogonal protecting group chemistry on defined pseudo-cysteines, but this is outside the scope of this discussion.

In summary, we have demonstrated that it is possible to install a reactive NCL component at will anywhere in a synthetic peptide and generate products with a variety of thioesters, including protein thioesters generated using intein chemistry. We have further demonstrated that by installing two reactive NCL components it is possible to link two different thioesters together. Finally, we have shown that there is no theoretical limit to the number of reactive NCL components installed in a peptide sequence, leaving the way open to use this chemistry to generate a chemical scaffold for the attachment of multiple other molecules in a stable, native, fashion.

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