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Native chemical ligation derived method for recombinant peptide/protein C-terminal amidation

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ABSTRACT

C-terminal amidation is often a requisite structural feature for peptide hormone bio-activity. We report a chemical amidation method that converts peptide/protein thioesters into their corresponding C-terminal amides. The peptide/protein thioester is treated with 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliary (**1b**) in a native chemical ligation (NCL) reaction to form an intermediate, which upon removal of the auxiliary with TFA, yields the peptide/protein amide. We have demonstrated the general utility of the approach by successfully converting several synthetic peptide thioesters to peptide amides with high conversion rates. Preliminary results of converting a recombinant peptide thioester to its amide form are also reported.

Many peptide therapeutics contain C-terminal amides that are necessary for their biological activity and/or for stability.[1] While short peptide amides can be conveniently made by solid phase synthesis, there is a need for scalable, cost-effective processes to produce long peptide/protein amides using recombinant procedures coupled with post-translationalamidation.

Enzymatic as well as chemical methods have been developed for C-terminal amidation. Peptidylglycine alpha-amidating monooxygenase (PAM),[1–4] the enzyme responsible for in vivo amidation of peptide hormone precursors, has found use in the recombinant manufacture of peptide hormones such as salmon calcitonin.[5] Transpeptidization reactions catalyzed by carboxypeptidase Y (CPD-Y) have also been described for amidation.[6–10] These methods often have limitations due to the enzymes' substrate specificities, special purification requirements as well as ready accessibility of the enzyme reagent. Chemical methods for amidation have also been reported such as chemical cleavage and amidation withpalladium[11] or cyanylation and aminolysis.[12–14] Yields or conversion rates are often poor or modest for chemical methods and are also sequence dependent.

Native chemical ligation (NCL) is a powerful synthetic approach to ligate native peptide/proteins in order to prepare longer peptide/proteins.[15–17] The original NCL method requires a peptide segment with a C-terminal thioester for coupling with a segment that has a cysteine at the N-terminus. Since the first report by Dawson and Kent,[16] many follow-on NCL methods have emerged to widen its applications. Auxiliaries such as 1-phenyl-2-mercaptoethyl[18] and its derivatives[19–21] were appended to the N-terminus of a peptide and used as surrogates of cysteine to ligate with a thioester peptide. The auxiliary can later be removed to reveal an unmodified peptide product. During our routine use of NCL to make C-terminal amidated bio-active peptides greater than sixty residues in length, we employed a 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliary reported by Macmillan et al.,[20] which permits cysteine-free ligation. The auxiliary **4a**, when installed at the N-terminus of one peptide to generate **1a**, provides a cysteine-like moiety to ligate to the peptide thioester. The auxiliary is then removed by TFA treatment to generate a longer peptide **3a** with a newly created amide bond Scheme 1.

This prompted us to investigate a variant of this auxiliary as a source of amide nitrogen to generate a C-terminal amidated peptide. The proposed reaction is also depicted in Scheme 1, where a peptide/protein thioester is treated with 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliary **1b** (1-(2,4-dimethoxyphenyl)-2-mercaptoethylamine) under NCL conditions to form an intermediate **2b**, which upon removal with TFA, yields the peptide/protein amide **3b**.

The auxiliary molecule **1b** is derived from Macmillan's auxiliary **4a**, but without the trityl protecting group, as we wished to employ the thiol functionality for direct ligation. We found that the dimerized form **5**, composed of a mixture of bothdiastereomers, is produced after iodination reaction to remove the trityl protection group from **4a** (Scheme 2). Dimer **5** can be reduced to generate **1b**. However, we elected to use **5** due to its stability and ease of purification. **5** was thus used in all the subsequent amidation

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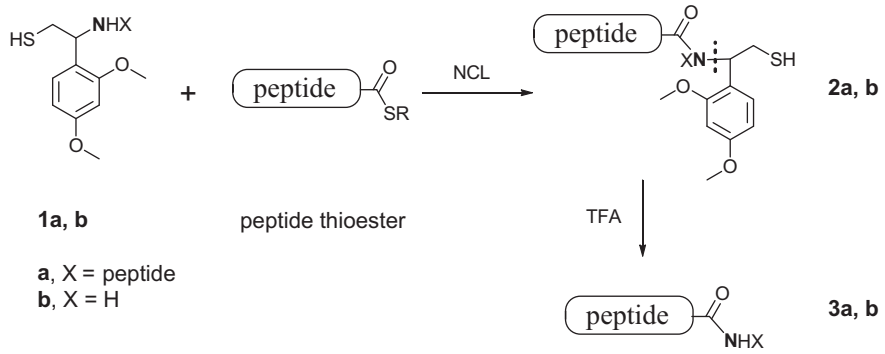
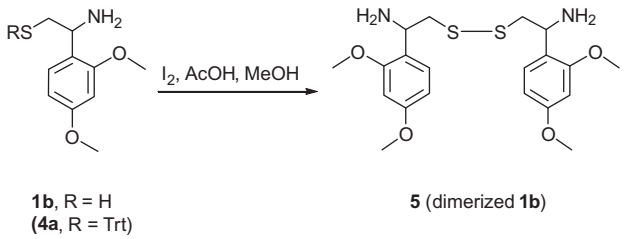


Scheme 1. General reaction scheme for the cysteine-free ligation reported by Macmillan et al. and native chemical ligation derived method for peptide/protein C-terminal amidation.

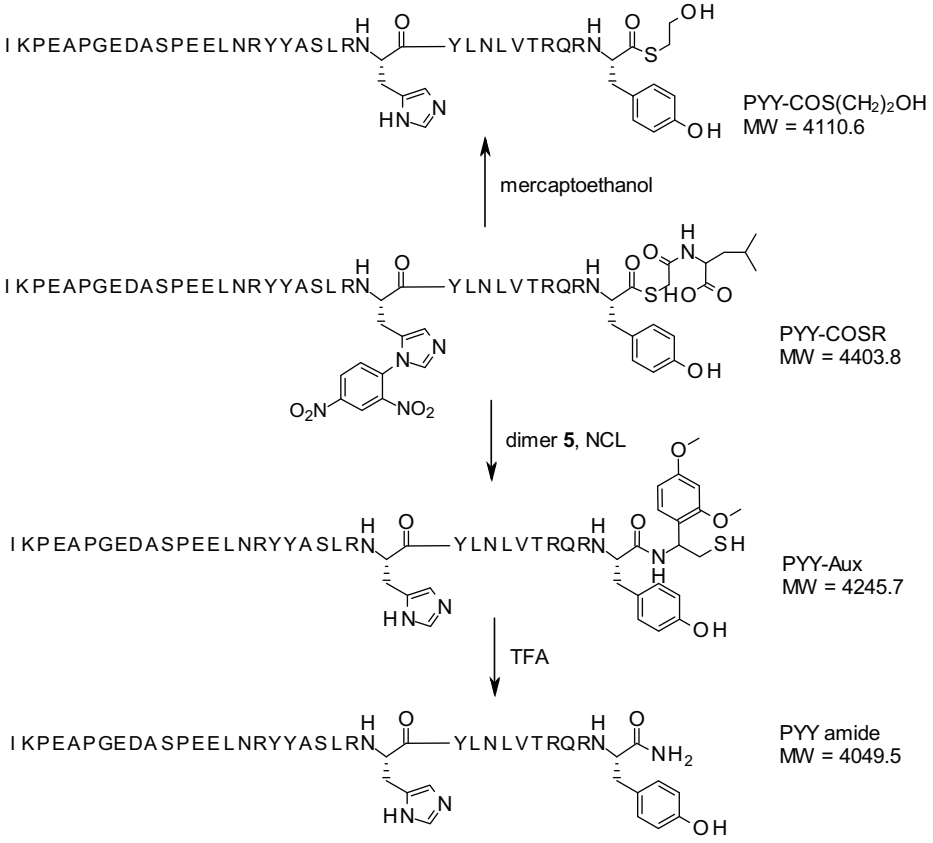


Scheme 2. Preparation of dimerized auxiliary **5** by iodination.

reactions, in which it was converted to **1b** in situ in the reductive native chemical ligation conditions.

We investigated the proposed amidation reaction with synthetic peptide thioester precursors of Davalintide (Dav),[22,23] PYY[3–36] (PYY)[24–27] and AC3174[28–30], an exendin-4 analog. Dav and PYY had been studied as clinical candidates for the treatment of obesity, and both peptides require an amidated C-terminus for biological activity. Exendin-4 is the active ingredient of Byetta® and BYDUREON®, and is also a C-terminal amidated peptide. The thioesters (Dav-COSR, PYY-COSR and AC3174-COSR, R = -CH_2CO-Leu-OH) were prepared by Boc Chemistry[31] using pre-loaded amino acid-SCH_2CO-Leu-OCH_2-Pam resin.[32] Peptide thioesters were cleaved using standard HF cleavage conditions with *p*-cresol as scavenger. The 2,4-dinitrophenyl (DNP) groups on histidines remained on the peptides and were removed by thiolysis during the native chemical ligation reaction.

PYY thioester (PYY-COSR) and auxiliary dimer **5** were ligated in a standard NCL reaction (6 M guanidine hydrochloride, pH 7.5 with 200 mM sodium phosphate and 20 mM TCEP, thiophenol 1.5 v/v%) for 3 h before quenching with mercaptoethanol. The reactions



Scheme 3. Amidation reaction of PYY thioester.