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# Native chemical ligation using removable $N^{\alpha}$-(1-phenyl-2-mercaptoethyl) auxiliaries

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**Abstract**—A new methodology to extend native chemical ligation is presented. This method makes use of a novel 1-phenyl-2-mercaptoethyl auxiliary moiety on the α-amino group of a first peptide segment, the auxiliary acts as a 1-amino-2-thiol-containing functional group to effect thioester-mediated, amide-forming ligation with a second thioester-containing peptide. Subsequent facile removal of the auxiliary from the newly formed amide gives products with only native peptide structures. © 2001 Published by Elsevier Science Ltd.

During the last few years thioester-mediated native chemical ligation (NCL) of unprotected peptide segments[1] has been demonstrated to be uniquely effective for the rapid and efficient chemical synthesis of numerous proteins.[2] Although several other ligation methods allow the chemoselective joining of two or more unprotected peptide fragments in aqueous solution,[3–5] only the NCL reaction leads to products with an unmodified peptide bond at the ligation site. The original NCL approach[1] has been routinely used to join two peptide segments at an Xxx-Cys sequence, where Cys is the N-terminal amino acid of the first segment and Xxx is an amino acid bearing an α-thioester at the C-terminal end of the second segment. The 1-amino-2-thiol arrangement of functionalities in the N-terminal Cys is essential for thioester-mediated amide-forming ligation.[1] Thus, NCL does not allow the ligation of unmodified polypeptide sequences devoid of suitably located cysteine residues.

Several attempts have been made to extend the principles of NCL to join peptides at other than Xxx-Cys ligation sites. Canne et al.[6] presented the first extension of the NCL reaction that made use of a $N^{\alpha}$-(thiol-containing) auxiliary group in lieu of the cysteine side chain thiol. The acid-stable, reductively cleavable $N^{\alpha}$-(oxyethanothiol) auxiliary of necessity had a 1-amino-3-thiol structure, and amide-forming ligation rates with this auxiliary were much slower, which has severely limited its practical utility. Canne et al. also tested an $N^{\alpha}$-(2-mercaptoethyl) ligation auxiliary that showed good rates of amide bond formation,[6] but the auxiliary was not designed for subsequent removal. A related method based on the same principle made use of $N^{\alpha}$-(2-mercaptobenzyl) as the auxiliary group, but this did not solve the problem of the removal of the auxiliary moiety after ligation.[7]

Here we present a novel, practical method that solves these problems and extends NCL to the joining of peptide segments at sites that do not contain cysteine residues. Our method makes use of the $N^{\alpha}$-(2-mercaptoethyl) auxiliary group and takes advantage of its fast ligation rates while allowing convenient and complete removal of the auxiliary after ligation. 1-Phenyl-substitution of the 2-mercaptoethyl auxiliary on the α-amino group of the reacting peptide segment gave a benzylamine derivative that is stable even under the strongly acidic conditions normally used in the synthesis of a peptide. The same group is readily cleaved under similar or even less acidic conditions when on the corresponding amide formed in the ligation reaction. Thus, a model peptide bearing either the $N^{\alpha}$-(1-phenyl-2-mercaptoethyl) auxiliaries **I** and **II** was synthesized using standard Boc chemistry solid phase peptide synthesis (SPPS) methods,[8] and reacted with a thioester-containing peptide. Ligation of the two segments gave the corresponding phenyl-substituted $N$-(1-phenyl-2-mercaptoethyl) amide. The auxiliary was then conveniently removed under acidic conditions to provide the native amide bond-linked polypeptide product. The general ligation strategy and auxiliaries employed in this approach are outlined in Scheme 1.

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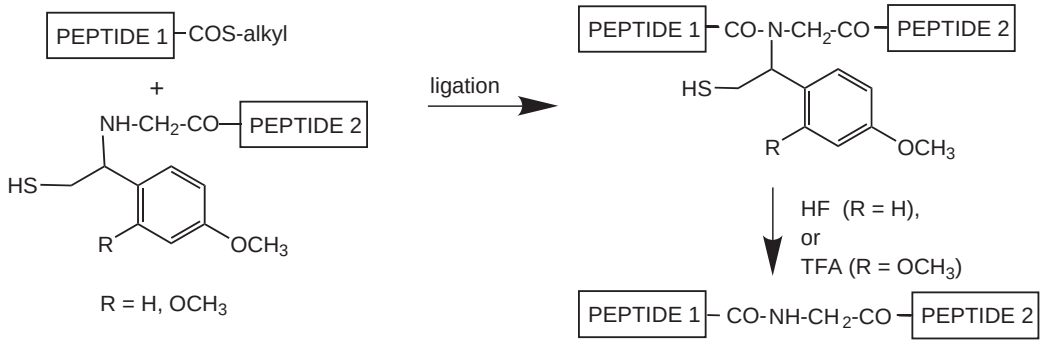


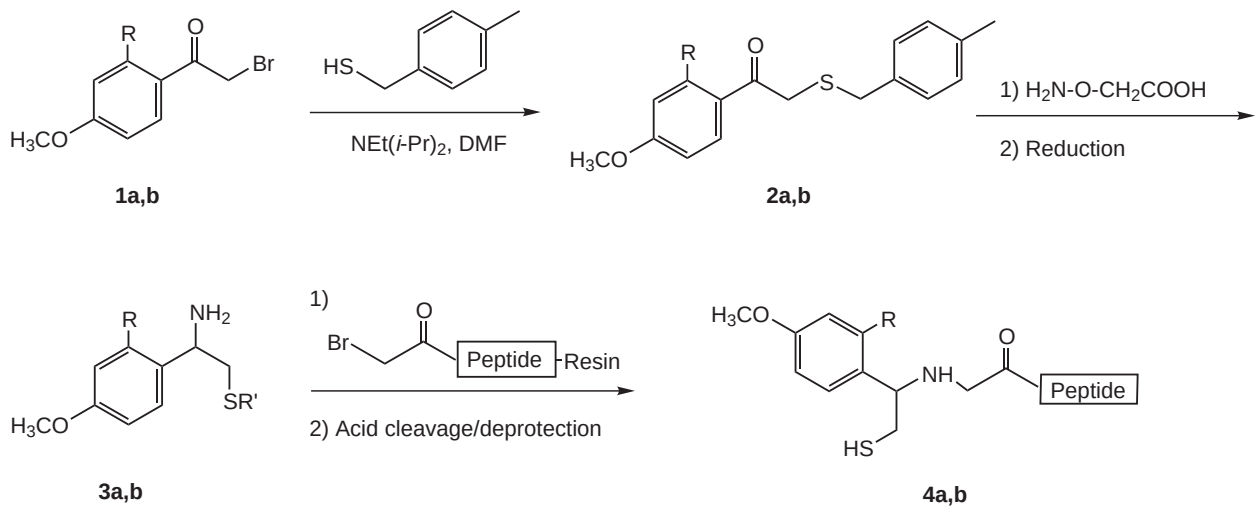

**Scheme 1.** General ligation strategy with 1-phenyl-2-mercaptoethyl auxiliaries.

The general synthesis of N-terminal glycine peptides containing the $N^{\alpha}$-(1-phenyl-2-mercaptoethyl) auxiliary is illustrated in Scheme 2. Two different benzylamines were prepared in three steps from the corresponding substituted bromoacetophenones: 1-(4-methoxyphenyl)-2-(4′-methylbenzylthio)-ethylamine (**3a**) and 1-(2,4-dimethoxyphenyl)-2-(4′-methylbenzylthio)-ethylamine (**3b**). Reaction of these amines with a resin-bound bromoacetylpeptide,[9] followed by cleavage/deprotection, gave peptides containing an N-terminal glycine residue derivatized with either an $N^{\alpha}$-(1-(4-methoxyphenyl)-2-mercaptoethyl) (**I**) or an $N^{\alpha}$-(1-(2,4-dimethoxyphenyl)-2-mercaptoethyl) (**II**) auxiliary.

The feasibility of using $N^{\alpha}$-(1-phenyl-2-mercaptoethyl) auxiliaries for native chemical ligation was tested with a model peptide of sequence $N^{\alpha}$(Aux)GlySerTyrArg-PheLeu. The N-terminus of the model peptide was derivatized with either the $N^{\alpha}$-(1-(4-methoxyphenyl)-2-mercaptoethyl) auxiliary (**I**), or the $N^{\alpha}$-(1-(2,4-dimethoxy-phenyl)-2-mercaptoethyl) auxiliary (**II**). Each of these peptides was successfully ligated with a variety of thioester peptides (Table 1). After purification of the ligation products, both auxiliary groups were efficiently removed using acidic conditions: the methoxy-substituted auxiliary **I** was removed with 95% HF and 5% *p*-cresol, or with trifluoroacetic acid (TFA)/bromotrimethylsilane (TMSBr);[10] while the dimethoxy-substituted auxiliary **II** was removed with 95% TFA, 2.5% triisopropylsilane (TIS), and 2.5% water. The removal of each auxiliary was accomplished in quantitative yield as measured by high performance liquid chromatography (HPLC).

The effectiveness of our new extended native chemical ligation strategy is highlighted by analytical HPLC data for model reaction 4 (Fig. 1). In this reaction, fragment 1-31 of Mouse Larc with a C-terminal Ala thioester was ligated with the model peptide $N^{\alpha}$(Aux)GlySerTyrArg-PheLeu bearing auxiliary **I**. After 40 h of ligation, the reaction was virtually complete. The ligation product was purified and then treated with {95% HF+5% *p*-cresol} at 0°C for 1 h, resulting in quantitative removal of the auxiliary **I**.

In summary, we have demonstrated a novel method that significantly extends the concept and practical utility of native chemical ligation. Moreover, the rates of ligation remained high, and the auxiliaries were easily removed. Importantly, our strategy is compatible with both Boc and Fmoc chemistries for peptide synthesis. The ability to join peptide fragments at sites other than Xxx-Cys sequences allows, inter alia, for convenient




**Scheme 2.** General synthesis of derivatized peptides (**a**: R = H; **b**: R = $OCH_3$; **R′** = *p*-Me benzyl).

**Table 1.** Results of ligation studies with $N^{\alpha}$(Aux)GlySerTyrArgPheLeu peptides

| Model reaction | Peptide-αCO-SR (C-terminal thioester residue) | $N^{\alpha}$-(Auxiliary) | Reaction time (h)[a] | Ligation yield (%)[b] | Auxiliary removal conditions |
|---|---|---|---|---|---|
| 1 | Phe-Gly-Gly | **I** | 16 | >98 | HF |
| 2 | Phe-Gly-Gly | **II** | 16 | >98 | TFA |
| 3 | TBP-A 1-67 (His) | **II** | 16 | 87 | TFA |
| 4 | Mouse Larc 1-31 (Ala) | **I** | 16 | 81 | |
| | | | 40 | 92 | HF |
| 5 | Mouse Larc 1-31 (Ala) | **II** | 16 | 73 | |
| | | | 40 | 85 | TFA |
| 6 | MCP1 1-35 (Lys) | **I** | 16 | 69 | |
| | | | 40 | 76 | TFA/TMSBr |

[a] Ligation conditions: 5 mM $N^{\alpha}$-(Aux) peptide in 6 M guanidinium phosphate buffer pH 7, 25°C, 2% thiophenol.
[b] Based on consumption of the $N^{\alpha}$-(Aux) peptide. Thioester peptides were used in 1.5-fold excess.

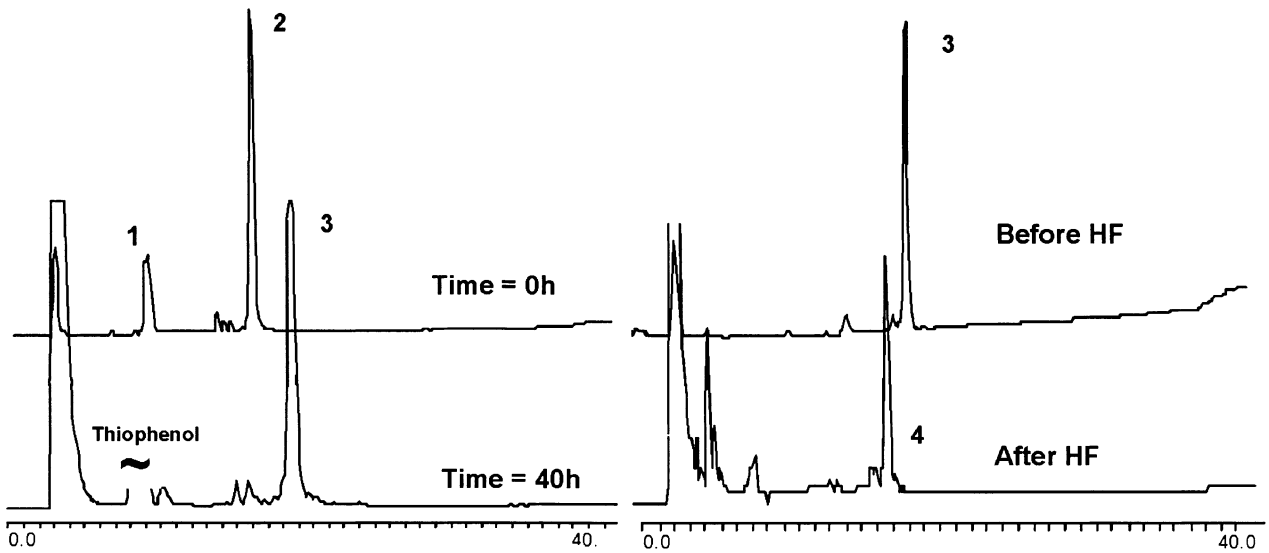



**Figure 1.** HPLC analyses of model ligation reaction 4 (Table 1). **Peak 1** = $N^{\alpha}$(Aux)Gly-SerTyrArgPheLeu; **peak 2** = Mouse Larc(1-31)αCOSR; **peak 3** = ligation product with $N^{\alpha}$-auxiliary; **peak 4** = HF crude from the removal of the $N^{\alpha}$-auxiliary moiety.

synthetic access to native, non-cysteine containing peptides and proteins. We will present data on the use of our 1-phenyl-2-mercaptoethyl auxiliary ligation strategy for the effective total synthesis of functional heme proteins.[11]

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