Tetrahedron Letters 51 (2010) 1793–1796

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Peptide ligation assisted by an auxiliary attached to amidyl nitrogen

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ARTICLE INFO

Article history:
Received 4 November 2009
Revised 25 January 2010
Accepted 29 January 2010
Available online 2 February 2010

ABSTRACT

New thiol-containing auxiliaries were developed for peptide ligation. They were placed at the amidyl N-atom in the second amino acid residue of a peptide fragment. With the new auxiliaries, peptide ligation could be conducted at non-Cys and non-Gly sites. Compared to other recently developed auxiliaries, an important feature of the present design was that the new auxiliaries were generally applicable and readily removable.

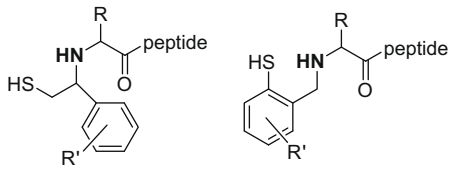
The efficiency of intramolecular catalysis due to the large increase of 'effective molarity' is a subject that has attracted escalating attention.[1] An elegant application of this strategy to bioorganic chemistry is the thiol-capture method of protein native chemical ligation (NCL) developed by Kent and co-workers.[2] NCL harnesses the high efficiency of intramolecular catalysis for the purpose of effecting amide ligation between peptide fragments. It employs an *S*-to-*N* acyl transfer reaction via a five-membered ring intermediate.[3]

Currently NCL and its related methods (e.g., expressed protein ligation) are extensively used for chemical protein synthesis.[4] An often encountered problem of NCL is that cysteine (Cys) is not always observed in all the natural proteins. To avoid the demand of Cys at the ligation site, several methods have been developed by attaching a thiol-containing auxiliary at the N-terminus of the peptide (Scheme 1).[5] A secondary amino group is generated in this type of auxiliary, leading to a relatively slow *S*-to-*N* acyl transfer. As a result, the ligation methods assisted by N-terminal auxiliaries can only be used for very few junctions such as Gly–Gly.

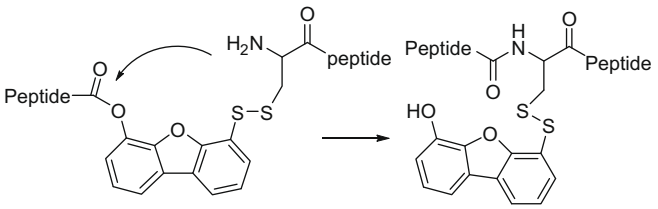
Alternatively one can also put the thiol-containing auxiliary at the other locations instead of the N-terminus. This strategy results in a larger ring size in the acyl transfer, whose efficiency may be lower than the five-membered ring acyl transfer in standard NCL. Nonetheless, Kemp et al. have shown that through strategic design it was possible to achieve an efficient acyl transfer through a medium-size ring intermediate (Scheme 2).[6] More recently, Wong and co-workers extended Kemp's method by anchoring an auxiliary to the carbohydrate moiety of a glycosylated amino acid to facilitate the *S*-to-*N* acyl transfer.[7] This method allows for the fragment condensation of glycopeptides at junctions containing neither Cys nor Gly. However, the use of a sugar auxiliary for ligation is expensive, and its application to the synthesis of sugar-free proteins is limited.

Herein, we describe a new design of the auxiliary for the thiol-capture method of peptide ligation. In the design the thiol-containing group is attached to the amidyl N-atom at the second amino acid residue of the peptide (Scheme 3). This design has several advantages over the previous ones: (1) The N-terminal group remains a primary amine. This may allow for the ligation at non-Gly sites. (2) Almost every peptide contains an amidyl N–H at its second amino acid residue. Thus the new design is more generally applicable as compared to e.g. sugar-assisted auxiliary.[7] (3) Many previous studies have shown that an auxiliary on an amidyl N-atom can be readily removed after ligation.[5]

To examine the effectiveness of the new design, we notice that the chemical structure of the spacer in the thiol-containing auxil-



Scheme 1. Auxiliaries at N-terminal nitrogen producing a secondary amine for acyl transfer.



Scheme 2. Kemp's ligation via a 12-membered ring intermediate.

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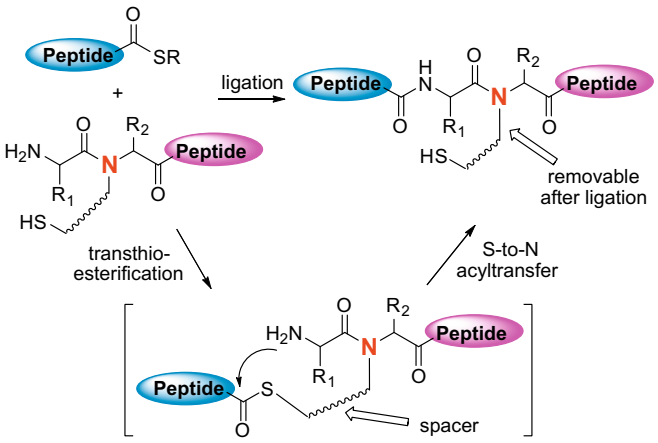
doi:10.1016/j.tetlet.2010.01.108



Scheme 3. The design of a new thiol-containing auxiliary.

iary remain to be optimized. Accordingly we have synthesized a series of model compounds **1a**–**1e** which all possess a thiol-containing side chain at their amidyl N-atoms (Fig. 1). The length (from two to four carbons) and nature (sp^3 or sp^2 carbons) of the spacer are systematically examined to probe their effects on the intramolecular acyl transfer. Scheme 4 outlines the synthesis of these model compounds by using **1e** as an example. An important step in the synthesis is the change of the protecting group on the thiol group from the trityl group to the *t*-butylthio group.[8]

The efficiency of the different spacers in the acyl transfer reaction was examined by the model ligation reactions carried out under normal NCL conditions (Scheme 5). It was found that with compound **1a**, **1c**, **1d**, and **1e**, the ligation yields were relatively low. Fortunately, when compound **1b** was used, the ligation yield reached 71%. Note that for **1b** to undergo the *S*-to-*N* acyl transfer, a nine-membered ring intermediate must be formed (Scheme 5). This observation supports the previous results that an efficient acyl transfer may proceed via a medium-size ring intermediate.[6,7,9]

The results from the above model study indicate that the amidyl auxiliary strategy is experimentally feasible and may constitute a new method for the bioorthogonal ligation. Our next tasks include: (1) to modify the spacer so that it can be removed after ligation; (2) to show that the new method can be applied to ligation at non-Cys and non-Gly sites; and (3) to further optimize the spacer to achieve

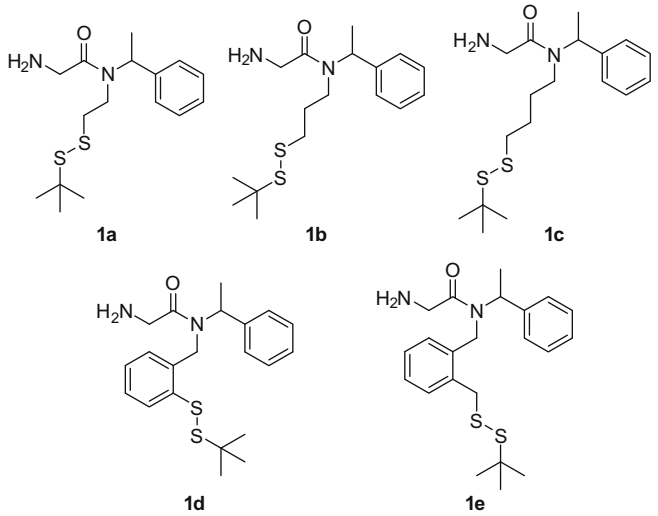
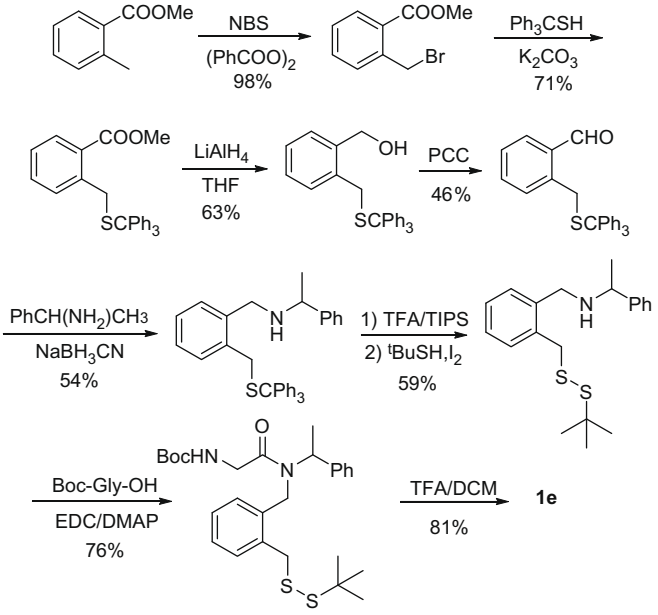
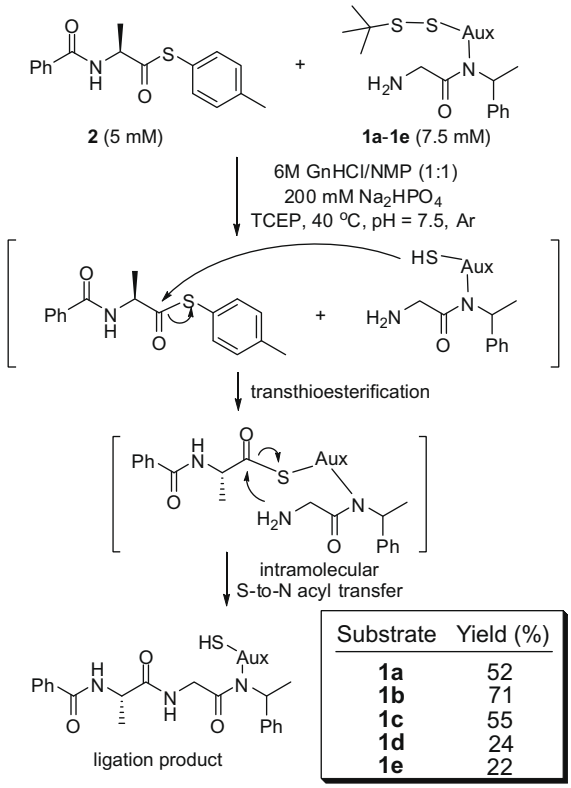


Figure 1. Model compounds possessing a thiol-containing side chain at their amidyl N-atoms.



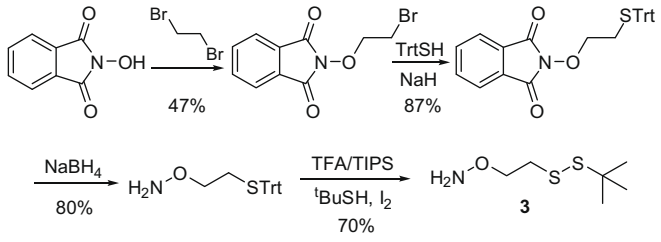
Scheme 4. Synthesis of compound **1e**.



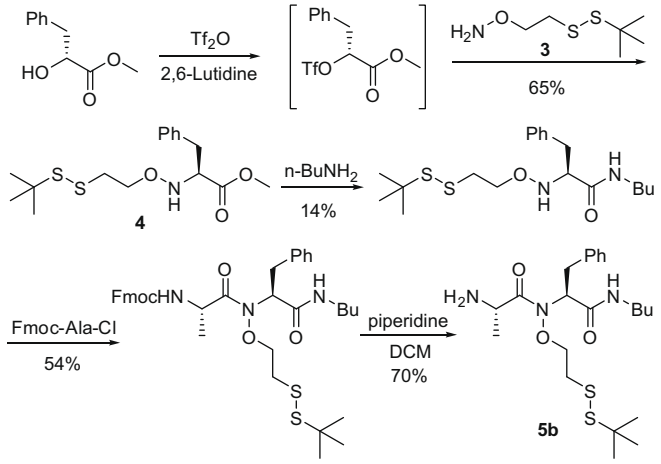
Scheme 5. Model ligation reactions with **1a**–**1e**.

higher ligation yields. In the following work we have solved the first two problems.

As to the first problem, our solution is to attach the oxyalkyl substitution on the amide bond. This substitution was previously examined by Kent and co-workers in their study on the ligation of N^{α}(oxyethanethiol)-peptide.[10] In Kent's study, the Boc-chemistry was utilized so that HF was used to deprotect the thiol in the preparation of N^{α}(oxyethanethiol)-peptide. In our study, we focus on the milder Fmoc-chemistry and therefore, we need to develop new synthesis to make the N^{α}(oxyethanethiol)-amino acid.

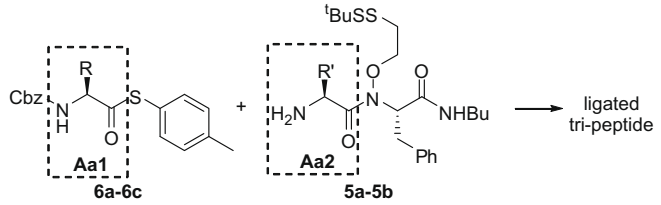


Scheme 6. Synthesis of compound **3**.



Scheme 7. Synthesis of the removable auxiliary.

Table 1
Ligation yields between different amino acid residues[a]



Entry	Aa1	Aa2	Yield[b] (%)
1	Gly	Gly	92
2	Ala	Gly	68
3	Val	Gly	58
4	Gly	Ala	66
5	Ala	Ala	45
6	Val	Ala	<10

[a] Conditions: **6** (5.0 mM), **5** (7.5 mM), TCEP (100 mM), 4:1 v/v NMP/buffer/GnHCl (6 M)/Na_2HPO_4 (0.2 M), pH 7.3–7.5, 40 °C.
[b] HPLC yield.

To this end, we have synthesized compound **3** as outlined in Scheme 6. From readily available α-hydroxycarboxylic acid (which can be made from the corresponding amino acid), we then obtain the desired N^{α}(oxyethanethiol)-amino acid **4** through a displacement reaction with inversion of stereochemistry (Scheme 7). With the amino acid residue **4** in hand, we can easily make the peptide. Noteworthily, Kent and co-workers have demonstrated that the oxylalkyl group on the amide bond can be selectively removed by facile treatment with Zn in acidic medium to give a native peptide after the ligation. Therefore, by using N^{α}(oxyethanethiol)-amino acid we can solve the first problem.

As to the second problem, we examined the ligation yields between different amino acid residues (Table 1). Gly, Ala, and Val were selected to represent amino acid residues with very low, modest, and very high steric hinderance, respectively. It was found that when both amino acids at the ligation site are glycines, the yield is as high as 92% (entry 1). When one of the two amino acids is glycine (either at the thioester side or the other side), the ligation yield is around 60–70% (entries 2–4). Importantly, when both amino acids are alanine, the ligation yield is 45% (entry 5). Therefore, the new type of auxiliary can allow for the ligation at a non-Cys and non-Gly site. This property is of importance because the earlier auxiliaries attached to the N-terminal nitrogens cannot be used for ligations at non-Gly sites under normal NCL conditions. Only the sugar-assisted auxiliaries and the present method can allow for ligations at non-Gly sites because in both cases the nucleophile in the *S*-to-*N* acyl transfer is a primary amine. Nonetheless, the ligation between Val and Ala exhibits a low yield in our study (entry 6), presumably because the steric hinderance in this particular case is too high.

At this point we have demonstrated that the new auxiliary can be attached to the peptide in a removable fashion. More importantly, the new auxiliary can allow for the ligation between non-Cys and non-Gly residues. Admittedly the ligation yields at non-Gly sites are relatively low. However, we believe that this problem can be solved by the next generation of spacer design in our ensuing study, in which we will incorporate a ring structure (e.g., a cyclohexanyl group like that in sugar-assisted auxiliary[7]) into the spacer.

To conclude, in this Letter we report the first generation of new thiol-containing auxiliaries that are placed at the amidyl N-atom in the second amino acid residue of a peptide fragment.[11–13] The experimental results provide proof of principle for the utility of the new auxiliaries that ligation can now be conducted at non-Cys and non-Gly sites with modest yields. Compared to other recently developed auxiliaries (e.g., sugar-assisted auxiliaries[7]), an important feature of the current design is that the new auxiliaries are generally applicable and readily removable. Further studies are in progress to evolve a second generation of removable, thiol-containing auxiliaries attached to the amidyl N-atom that allows for ligation at non-Cys and non-Gly sites with more satisfactory yields.

Acknowledgments

This work was supported by NSFC (Nos. 20802040 and 20932006), and the Specialized Research Fund for the Doctoral Program of Higher Education of China (Grant No. 200800030074).

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11. *Compound* **1a**: HR-ESI *m*/*z*: 327.15584 $[M+H]^+$ (calcd for $C_{16}H_{26}N_2OS_2$ 326.52044); *compound* **1b**: HR-ESI *m*/*z*: 341.17154 $[M+H]^+$ (calcd for $C_{17}H_{28}N_2OS_2$ 340.54702); *compound* **1c**: HR-ESI *m*/*z*: 355.18703 $[M+H]^+$ (calcd for $C_{18}H_{30}N_2OS_2$ 354.57360); *compound* **1d**: HR-ESI *m*/*z*: 389.17174 $[M+H]^+$ (calcd for $C_{21}H_{28}N_2OS_2$ 388.58982); *compound* **1e**: HR-ESI *m*/*z*: 403.18720 $[M+H]^+$ (calcd for $C_{16}H_{26}N_2OS_2$ 402.61640).
12. Ligation product from compound **1b** of Scheme 5: ESI-MS *m*/*z*: 450.4 $[M+Na]^+$ (calcd for $C_{23}H_{29}N_3O_3S$ 427.2).
13. Products of Table 1. Entry 1: ESI-MS *m*/*z*: 545.2 $[M+H]^+$ (calcd for $C_{27}H_{36}N_4O_6S$ 544.2); entry 2: ESI-MS *m*/*z*: 559.2 $[M+H]^+$ (calcd for $C_{28}H_{38}N_4O_6S$ 558.3); entry 3: ESI-MS *m*/*z*: 587.1 $[M+H]^+$ (calcd for $C_{30}H_{42}N_4O_6S$ 586.3); entry 4: ESI-MS *m*/*z*: 559.3 $[M+H]^+$ (calcd for $C_{28}H_{38}N_4O_6S$ 558.3); entry 5: ESI-MS *m*/*z*: 573.3 $[M+H]^+$ (calcd for $C_{29}H_{40}N_4O_6S$ 572.3).