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Review

Design of thiol-containing amino acids for native chemical ligation at non-Cys sites

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ABSTRACT

Protein chemical synthesis usually relies on the use of native chemical ligation that couples peptide thioester with a Cys-peptide. A limitation of this method is the difficulty of finding an appropriate Cys ligation site in many synthetic targets. To overcome this problem, the ligation–desulfurization approach has been developed. This approach involves the use of a thiol-containing amino acid as the ligation partner. After the sequence assembly is completed, the thiol group is removed through a desulfurization reaction to generate the standard amino acids. Currently this strategy has been applied to the ligations at a number of amino acids including Ala, Phe, Val, Lys, Thr, Leu, Pro and Gln. The present article reviews the design and synthesis of these thiol-containing amino acids for native chemical ligation at non-Cys sites.

1. Introduction

In the past two decades the native chemical ligation method has been established as the most effective approach for the chemical synthesis of proteins [1,2]. It has a number of advantages such as the employment of fully unprotected protein fragments for the ligation and the use of neutral aqueous buffers as reaction media. Native chemical ligation basically involves a chemoselective coupling reaction of a peptide thioester with a Cys-peptide, through which a peptide bond is generated with high efficiency. An often encountered limitation of the native chemical ligation approach is the need for a Cys residue at the ligation site. Because the natural abundance of Cys is only 1.7% among all the amino acids [2], it is often difficult to find a Cys residue in the synthetic target, or the Cys residue is located at a position not suitable for the design of peptide fragments.

To solve the above problem many groups have developed methods to avoid the requirement of a Cys residue in the ligation. These methods include the attachment of an auxiliary thiol group to *N*-terminal amino acid [3], the use of selenocysteine-based ligation [4], and the employment of a different type of ligation reactions [5]. Through the application studies it has been recognized that among all these approaches, the most effective one is the attachment of a thiol group to the side chain of the *N*-terminal amino acid (Fig. 1). This method best keeps the feature of *S*-to-*N* acyl shift in the native chemical ligation reaction, so that its efficiency is high. After the ligation, the thiol group can be chemically removed to regenerate the original *N*-terminal amino acid. In the present article we review the progress that has been made for the abovementioned ligation–desulfurization approach. Our emphasis is the design and synthesis of the thiol-modified amino acids.

2. Ligation at Ala

The first amino acid that was tested for the ligation–desulfurization strategy is Ala, because after attachment of a thiol group at its side chain Ala becomes Cys. Thus, the standard native chemical ligation between a peptide thioester and a Cys-peptide can be carried out first. Subsequently we remove the thiol group from the ligated product, and Cys will be converted back to the original Ala residue. An important advantage of this approach is that Ala is much more abundant than Cys in proteins. Therefore, the above approach greatly expands the scope of native chemical ligation.

In 2001 Dawson and Yan first studied the above method and used it in the synthesis of an antibiotic named Microin J25 [6]. This molecule is a cyclic peptide containing 21 amino acids. It contains Ala but not Cys. To synthesize this molecule, Dawson *et al.* first mutated Ala to Cys and prepared the corresponding linear peptide with an *N*-terminal Cys and a C-terminal Gly thioester. Through native chemical ligation this linear peptide was successfully

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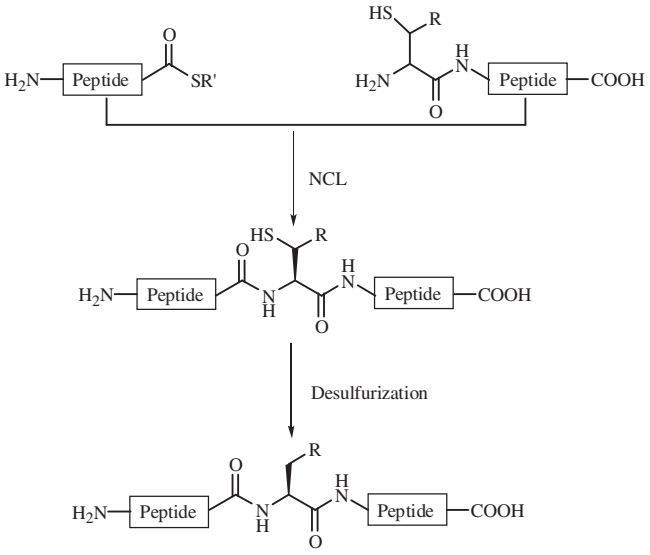
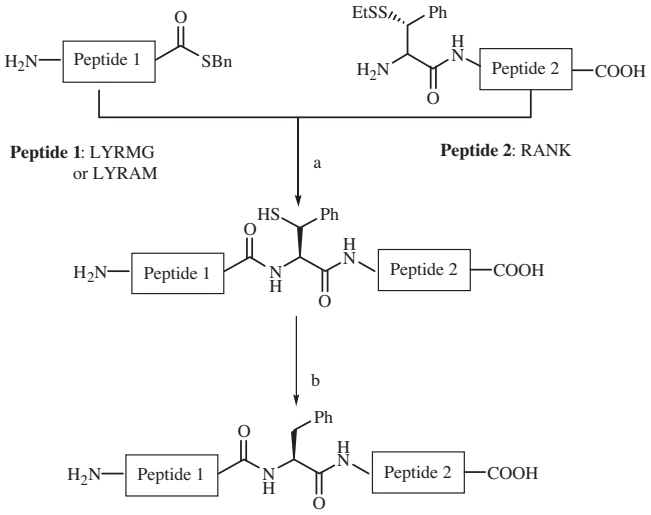


Fig. 1. The ligation–desulfurization approach in protein chemical synthesis.



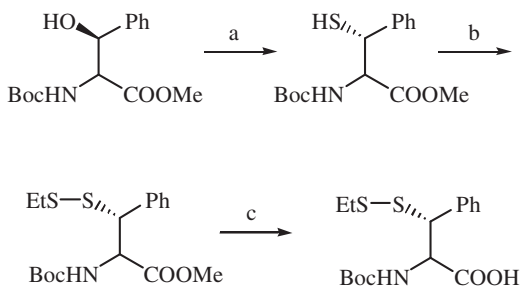
Scheme 2. Ligation at thiol-containing Phe. (a) MESNa, TCEP, 0.1 mol/L Tris–HCl, pH 8 and (b) $NiCl_2{\cdot}6H_2O$, $NaBH_4$, 0.1 mol/L phosphate buffer, pH 7.

cyclized. Then the Cys residue was converted to Ala by hydrogenation over Pd/Al_2O_3 in a neutral aqueous buffer. Note that Pd/C, $Pd/BaSO_4$, and Ni catalysts can also be used for the desulfurization step.

The above transition metal-catalyzed desulfurization method has also been successfully used by Kent and Pentelute in the synthesis of proteins including EETI-II [7] and ubiquitin [8]. However, it was also recognized that metal catalysts may cause the adsorption and loss of peptides as well as some side reactions (*e.g.* desulfurization of Met). To overcome this problem Danishefsky and Wan developed a free-radical based desulfurization approach [9]. In this method, VA-044 was used as the radical initiator and TCEP was used as the reducing reagent. This approach can be used to remove the thiol group with fairly high yields and selectivity [10].

3. Ligation at Phe

In 2006 Crich and Banerjee extended the ligation–desulfurization method to Phe [11]. They first synthesized thiol-containing Phe through four steps (Scheme 1). After attaching this amino acid to the N-terminus of a synthetic peptide, they successfully carried out ligation with a peptide thioester (Scheme 2). The resulting intermediate was then treated with $NaBH_4/NiCl_2$ to remove the thiol group. The target peptides (*i.e.* YRMG-**F**RANK and LYRAM-**F**RANK) that contained no Cys in their sequences were obtained in 60% and 57% yields, respectively.

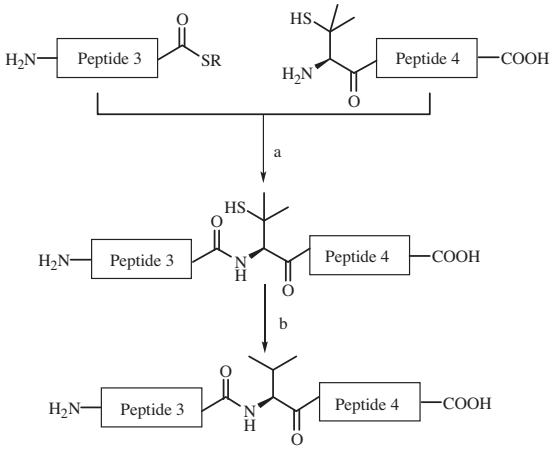


Scheme 1. Synthesis of thiol-containing Phe. (a) (i) MsCl, Et_3N, CH_2Cl_2; (ii) AcSH, DBU, DMF and (iii) NaOH/MeOH; (b) EtS-SOEt, Et_3N, CH_2Cl_2 and (c) LiOH/THF.

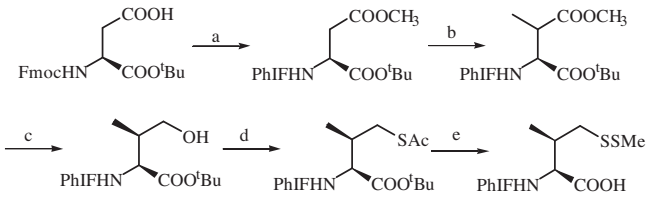
4. Ligation at Val

In 2008 Seitz *et al.* used commercially available penicillamine as a thiol-containing amino acid to accomplish the ligation at Val [12] (Scheme 3). Through experiments they found that this thiol-containing amino acid can be successfully ligated with peptide thioesters with a C-terminal amino acid of Gly, His, Met, and even Leu. This observation is fairly surprising because one may expect a high steric hindrance of the two neighboring methyl groups. Furthermore, the thiol group can be efficiently removed by using the free-radical based desulfurization approach. By using the above method, Seitz *et al.* successfully synthesized fragments of kinases STAF1 and Syk. The ligation yields were 78% and 87%, whereas the desulfurization yields were 72% and 98%.

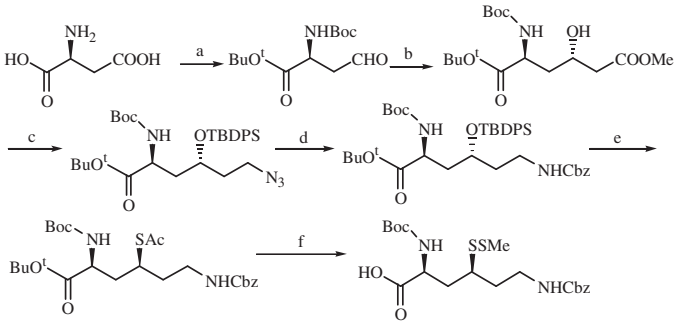
In the same year Danishefsky *et al.* designed a different route for the synthesis of thiol-containing Val [13] (Scheme 4). They started from Fmoc-L-Asp and successfully attached the thiol group at the side chain methyl of Val. Due to the lower steric hindrance of the new molecule as compared to penicillamine, both the ligation rate and yield were improved. For instance, in the native chemical ligation for the preparation of Fmoc-Thz-RGDSCys(Acm)RPG**Q**-**V**GAPRHSWG-OH, the use of the new molecule enabled the ligation



Scheme 3. Ligation at thiol-containing Val. (a) 100 nmol/L sodium phosphate buffer (0.6 mol/L GnHCl, 50 mmol/L TCEP, 5% PhSH), pH 8.5, 37 °C and (b) 100 mmol/L sodium phosphate buffer (3 mol/L GnHCl, 200 mmol/L VA-044, 250 mmol/L TCEP, 40 mmol/L glutathione) pH 6.5, 65 °C.



Scheme 4. Synthesis of thiol-containing Val. (a) (i) $TMSCHN_2$, ether, MeOH; (ii) diethylamine, DMF, CH_2Cl_2 and (iii) 9-bromo-9-phenylfluorene, K_3PO_4, CH_3NO_2; (b) KHMDS, MeI, THF, −78 °C; (c) DIBAL-H, THF, −35 °C; (d) (i) MsCl, Et_3N, CH_2Cl_2; (ii) AcSH, DBU, DMF and (e) (i) NaOH, MeOH, 0 °C; (ii) MMTS, Et_3N, CH_2Cl_2; (iii) HCl/EtOH.



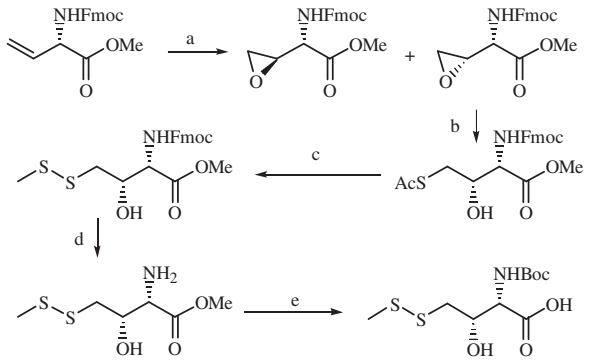
Scheme 5. Synthesis of thiol-containing Lys. (a) (i) CH_3OH, HCl; (ii) $(Boc)_2O$, Na_2CO_3; (iii) DCCI, DMAP, *t*BuOH, CH_2Cl_2; (iv) 1 mol/L NaOH, acetone/H_2O; (v) EtSH, DCC, DMAP, DCM and (vi) EtSiH, 10% Pd/C, DCM; (b) methyl bromoacetate, Zn, TMSCl; (c) (i) TBDPSl, imidazole, DCM; (ii) $NaBH_4$, EtOH; (iii) methanesulfonyl chloride, DIPEA and (iv) NaN_3, DMF; (d) (i) Pd/C, ethyl acetone and (ii) CbzCl, $NaHCO_3$, dioxane/water (2:1); (e) (i) TBAF, THF; (ii) methanesulfonyl chloride, DIPEA and (iii) potassium thioacetate, DMF and (f) (i) NaOH, MeOH; (ii) *S*-methyl methanethiosulfonyl chloride, triethylamine, CH_2Cl_2; (iii) 95% TFA/H_2O and (iv) $(Boc)_2O$/TEA, MeOH.

in 1 h with a yield of 80%, whereas the use of penicillamine required 9 h to complete the ligation with 66%. Further experiments showed that more difficult ligation sites such as Thr–Val and Pro–Val can be handled by the new thiol-modified Val.

5. Ligation at Lys

In 2009, Liu *et al.* accomplished the native chemical ligation at Lys residue [14]. They designed and synthesized a Lys derivative containing a thiol group at the γ-carbon (Scheme 5). This thiol-modified Lys can undergo ligations at both of its α-NH_2 and ε-NH_2 groups for the generation of peptide as well as isopeptide bonds. After the ligations are completed, the thiol group can be removed by the free-radical desulfurization approach. By using this strategy, Liu *et al.* successfully prepared Lys-ubiquitinated proteins [15]. They initially tested the use of Cbz group for the protection of the ε-NH_2, but found its removal required the employment of a strong acid. Later on they used a photolabile protecting group to overcome the above problem [16].

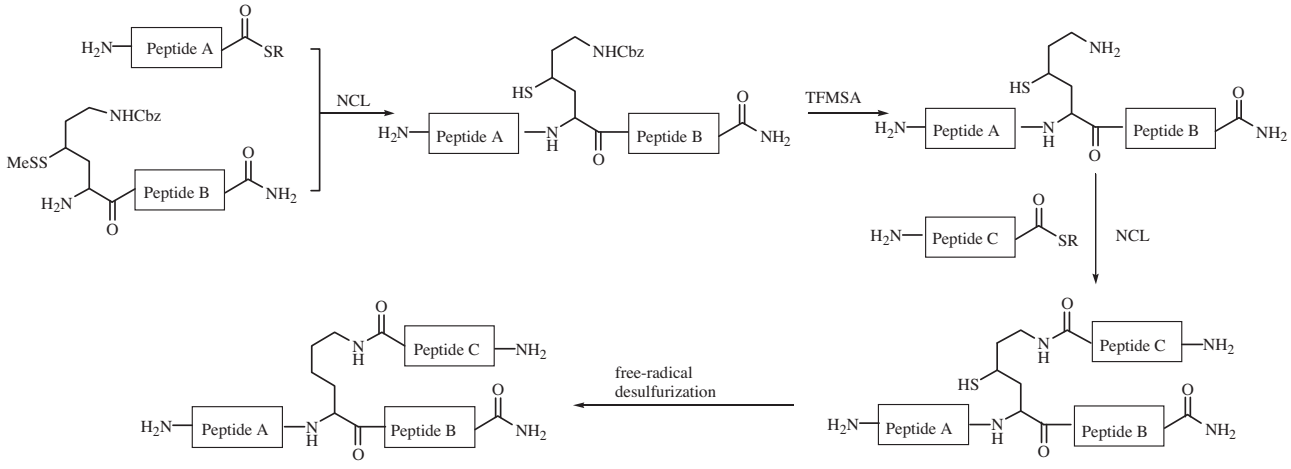
In 2009, Brik *et al.* also used the thiol-containing Lys for the synthesis of ubiquitinated proteins [17] (Scheme 6). They inserted it into a peptide sequence with a Thz at the N-terminus. Through the ligation at the Lys ε-NH_2 position with Ub(1–76)-α-thioester, they successfully attached ubiquitin to the peptide with an isopeptide bond. This ligation was completed in 4 h with a yield of 75%. Subsequently they converted the Thz residue to Cys. This Cys residue was further ligated with another peptide thioester to finish the assembly of the whole protein. The thiol groups were then removed by using the free-radical desulfurization approach.



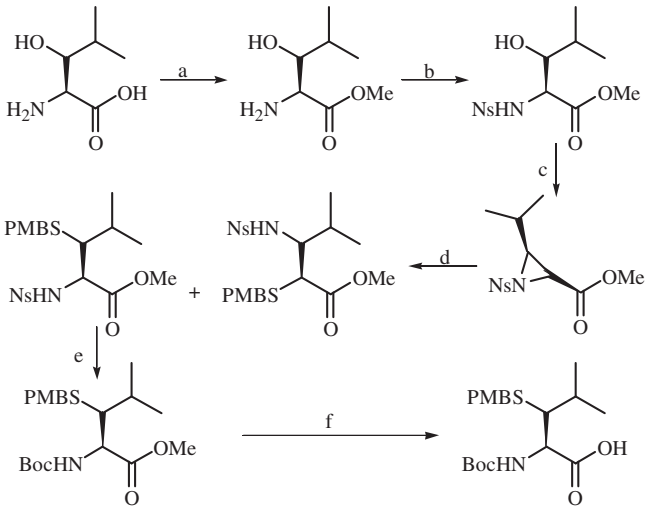
Scheme 7. Synthesis of thiol-containing Thr. (a) mCPBA, CH_2Cl_2; (b) CH_3COSH, CH_3COONa, toluene, DMF; (c) (i) 0.2 mol/L NaOH, MeOH, 0 °C and (ii) MMTS, DIEA, CH_2Cl_2; (d) DEA, DMF; (e) (i) $(Boc)_2O$, THF/MeOH/TEA; (ii) 1 mol/L NaOH, H_2O/THF.

6. Ligation at Thr

In 2010 Danishefsky *et al.* designed the thiol-containing Thr [18] (Scheme 7). Starting from D-vinylglycine, they successfully prepared the target compound in five steps. The application of this thiol-containing Thr in native chemical ligation was tested with peptide thioesters bearing different C-terminal amino acids. It was found that the steric hindrance of the C-terminus can significantly affect the efficiency of the ligation. By using the thiol-containing Thr, Danishefsky *et al.* successfully synthesized several glycopeptides.



Scheme 6. Use of thiol-containing Lys for the synthesis of ubiquitinated proteins.



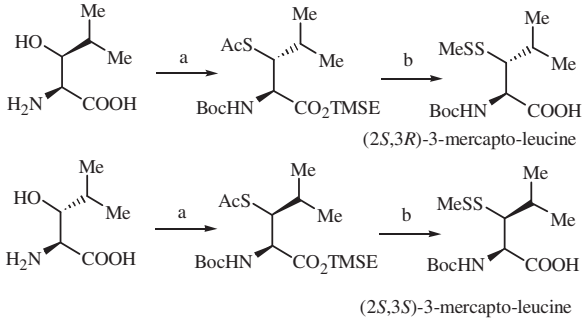
Scheme 8. Synthesis of thiol-containing Leu. (a) MeOH, $SOCl_2$; (b) *p*-nitrophenylsulfonyl chloride, Et_3N/CH_2Cl_2; (c) PPh_3, THF, diisopropyl-azodicarboxylate; (d) *p*-$MeOC_6H_4CH_2SH$, $BF_3{\cdot}OEt_2$, CH_2Cl_2; (e) (i) *p*-$MeOC_6H_4SH$, K_2CO_3, MeCN/DMSO and (ii) $(Boc)_2O$, $(i\text{-}Pr)_2EtN$, CH_2Cl_2 and (f) 1 mol/L LiOH, THF/H_2O.

The thiol group was eventually removed by using the free-radical approach with yields as high as 96%.

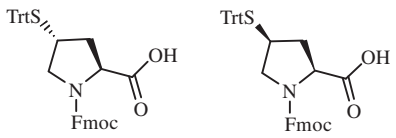
7. Ligation at Leu

In 2010 Brik *et al.* accomplished the use of thiol-containing Leu in native chemical ligation [19]. They first designed and synthesized the thiol-modified Leu (Scheme 8). Subsequently they examined its ligation with different amino acid thioesters including Gly, Ala, Ser and Leu. The yields ranged from 70% to 90%. Finally, they showed that the thiol group can be smoothly removed by using the free-radical desulfurization approach. By using the thiol-containing Leu, Brik *et al.* successfully synthesized a model protein HIV-1 Tat(1–86).

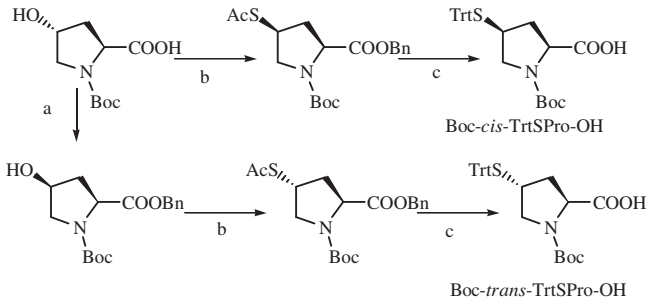
In the same year Danishefsky *et al.* also studied the native chemical ligation at Leu [20] (Scheme 9). They designed and synthesized two different stereoisomers and found that the (2*S*, 3*R*) isomer was more effective for the ligation. As to the thioesters of different amino acids, they found that the ligation with Gly, Ala, and Phe can be completed in about 2.5 h with yields over 85%. On the other hand, the ligation with sterically more demanding amino acids such as Pro and Val was much less efficient with yields ranging from 20% to 50%. By using the thiol-containing Leu, they also synthesized a model protein hPTH [21].



Scheme 9. Synthesis of another thiol-containing Leu. (a) (i) Boc_2O, Na_2CO_3, THF/H_2O; (ii) TMSE-OH, DCC, DMAP, CH_2Cl_2; (iii) MsCl, Et_3N, CH_2Cl_2 and (iv) AcSK (excess), DMF and (b) (i) NaOH, MeOH; (ii) MMTS, DIEA, CH_2Cl_2; (iii) TBAF, THF.



Scheme 10. Two commercially available thiol-containing Pro.

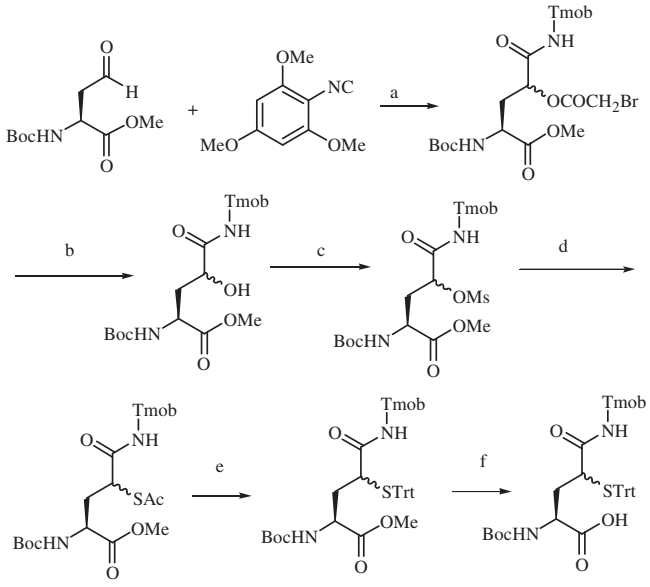


Scheme 11. Synthesis of thiol-containing Pro. (a) (i) DEAD, PPh_3, CH_2Cl_2; (ii) NaOH, THF/H_2O and (iii) BnBr, Et_3N, DMF; (b) (i) MsCl, Et_3N, CH_2Cl_2 and (ii) AcSK, DMF and (c) (i) NaOH, THF/H_2O and (ii) Trt-Cl, pyridine.

8. Ligation at Pro

In 2011, Danishefsky *et al.* examined the use of two commercially available thiol-containing Pro's in the native chemical ligation [22] (Scheme 10). It was found that the *trans* isomer exhibits a higher ligation efficiency. Only sterically less hindered amino acids such as Gly and Phe can be tolerated at the ligation site, whereas the ligation with the thioesters of Val and Pro leads to almost no desired product. By using the thiol-containing Pro, Danishefsky *et al.* successfully prepared a 166-amino acid protein EPO [23].

Furthermore, by using Boc-HOPro-OH as the starting material, Otaka et al. prepared Boc-*trans*-TrtSPro-OH and Boc-*cis*-TrtSPro-OH [24] (Scheme 11). In agreement with Danishefsky's study, Otaka *et al.* also found that the *trans* isomer is more reactive in the



Scheme 12. Synthesis of another thiol-containing Gln. (a) $BrCH_2COOH$, CH_2Cl_2; (b) thiourea, $NaHCO_3$, 50 °C; (c) MsCl, Et_3N, CH_2Cl_2, 0 °C; (d) CH_3COSH, DBU, DMF; (e) (i) 1 mol/L NaOH, MeOH, 0 °C and (ii) Trt-Cl, Et_3N, CH_2Cl_2; (f) 0.3 mol/L LiOH, THF: H_2O = 4:1, 0 °C.

ligation. By using Boc-*trans*-TrtSPro-OH, they also synthesized bovine insulin C peptide.

9. Ligation at Gln

In 2012, Brik *et al.* designed and synthesized thiol-containing Gln [25] (Scheme 12). This amino acid was obtained and used as a mixture of stereoisomers with regards to the thiol group, because after desulfurization both isomers will end up with the same Gln residue. The ligation of the thiol-containing Gln with four model peptide thioesters (*i.e.* LYRAG-SR, LYRA-SR, LYRAQ-SR, and LYRAL-SR) shows that the expected product can be obtained in good yields (70%, 62%, 56%, and 50%).

10. Conclusions and outlook

Since the invention of the ligation–desulfurization approach, a number of thiol-containing amino acids have been designed and synthesized. The use of these thiol-containing amino acids in the native chemical ligation has been shown to be successful, which greatly expands the scope and utility of native chemical ligation. So far the above strategy has been examined for Ala, Phe, Val, Lys, Thr, Leu, Pro, and Gln. We expect that future studies will be carried out for the other amino acids including Ile, Glu, Trp, Met, Tyr, Arg, and His. It would be difficult to install a thiol group in the side chain of Ser, Asn, and Asp. We expect that some alternative approaches will be developed to solve the problem [26]. Finally, although many thiol-containing amino acids have been made, their synthetic routes are relatively tedious. We expect that more efficient synthesis of these important amino acids will be developed in the near future [27].

Acknowledgment

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