Synthesis of Methionine Containing Peptides Related to Native Chemical Ligation

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Abstract: Methionine chemical ligation is based on a convenient direct thioester formation at the C-terminus of the ligation site, on homocysteine reduction with dithiothreitol in order to liberate the mercapto group of the homocysteine residue at the N-terminus, and on chemoselective S-methylation with methyl iodide.

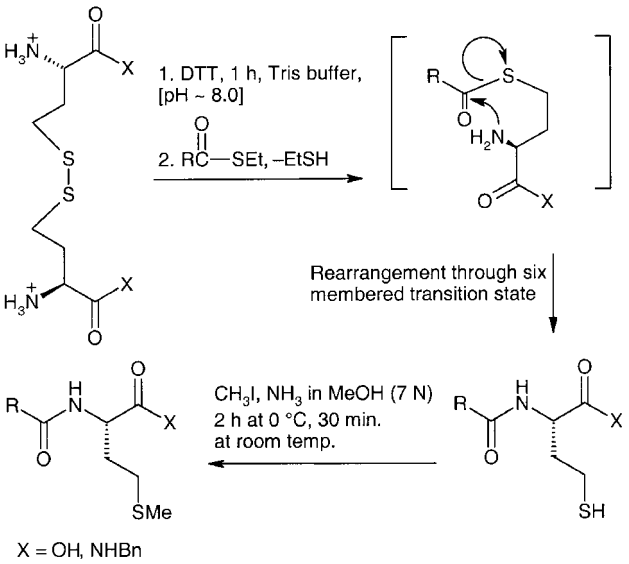
Key words: chemical ligation, methionine ligation, peptides, *S*-methylation

Solid phase peptide synthesis in combination with 'native chemical ligation' of unprotected peptides has gained enormous interest.[1] The development of native chemical ligation has opened the door to the synthesis of fully unprotected peptides and even proteins. Although native chemical ligation was already found by Wieland et al.[2] in 1953, its potential in the synthesis of a variety of proteins[1a,3] including cyclic proteins[4] was recognized only in recent times.

The native chemical ligation involves a simple mixing of two peptide segments under aqueous conditions in which one peptide contains a thioester unit at the C-terminus and the other one a cysteine residue at the N-terminus. The first step involves formation of a thioester-linked intermediate and the second step rearrangement through a five-membered transition state to form a native peptide bond.

Hence, a common required element is a cysteine residue at the ligation site. However, this requirement is also a limitation in terms of general applicability. Therefore, efforts have been made to extend the applicability of native chemical ligation to non-cysteine based ligations by employing N^{α}-oxyethanethiol[5] or N^{α}-thiobenzyl linker[6] as an auxiliary at the ligation site. These auxiliaries have to be removed after the ligation, in a separate step. Also other approaches for the synthesis of non-cysteine containing proteins were reported.[7,8]

Chemical ligation of a thioester at a peptide C-terminus with a homocysteine (Hcy) residue at the N-terminus of a second peptide is an interesting alternative; thus, after S,N-acyl migration through a six-membered transition state, a selective homocysteine S-methylation is required in order to introduce a methionine residue at the ligation site (Scheme 1).



Scheme 1 Peptide bond formation via a six-membered transition state and chemoselective methylation of the homocysteine thiol group

This reaction could be successfully performed with long peptides, however, in the ligation step three byproducts were detected and in the methylation step long reaction times led to overmethylation.[9] In this paper, the methionine chemical ligation is based on (i) convenient direct thioester formation at the C-terminus of the ligation site, (ii) in situ homocystine reduction with dithiothreitol (DTT), thus avoiding the observed byproduct formation in the ligation step, and (iii) chemoselective S-methylation with methyl iodide which is less reactive than previously employed methyl *p*-nitro-benzenesulfonate, thus restricting overmethylation.

Thioester **1**, and for further studies, thioesters **3**–**7** (Table 1) were readily prepared by coupling of the amino acids with ethanethiol in the presence of HOBt/DCC in DMF at room temperature. Under these conditions thioesters **1** and **3**–**7** were directly obtained from the corresponding amino acids in one step without protecting other nucleophilic centres such as hydroxy, amide, or heterocyclic groups (isolated yields 70–82%). Thioester **2** was prepared by deprotecting *N*-Boc protected **1** using 20% TFA in dichloromethane.

Reaction of homocysteine, generated in situ by reduction of homocystine using DTT as a reducing agent, with *N*-Boc protected thioester **1** in tris buffer (pH ca. 8.0) produced the desired dipeptide bearing a free thiol group in

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quantitative yield (Table 1). The thiol group was then methylated using methyl iodide in ammonia in methanol affording the expected Boc-Ala-Met dipeptide **8** which was isolated as free acid in 84% yield.[10,11] Byproduct formation was not observed. One-pot reaction of homocystine, DTT, thioester **1** and methyl iodide in buffer solution at room temperature also produced **8** albeit in lower yield.

Reaction of unprotected alanine thioester **2** with homocysteine yielded quantitatively the Ala-Hcy ligation product and chemoselective methylation of the thiol intermediate produced S-methylated AlaMet dipeptide **9** in 76% isolated yield.[10,11]

Table 1 Reaction of Homocysteine with Aminoacids and Peptide Thioesters Followed by S-Methylation

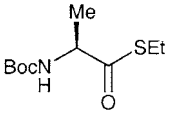
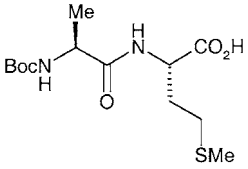
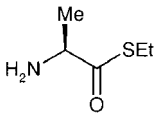
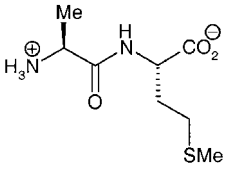
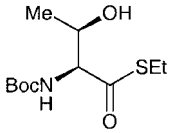
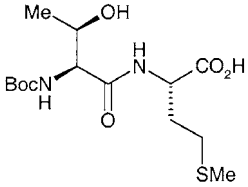
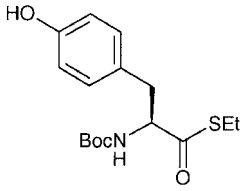
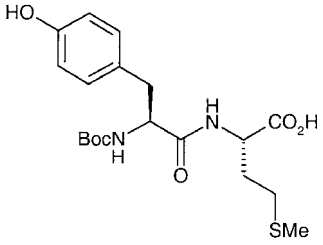
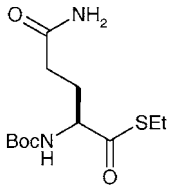
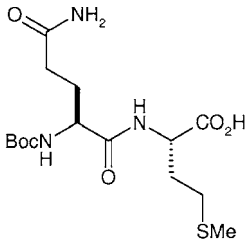
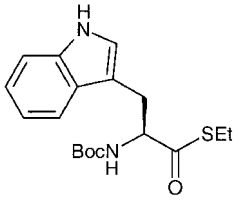
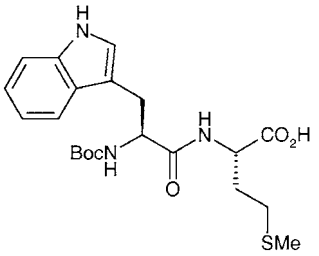
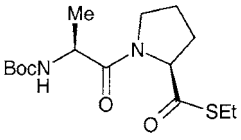
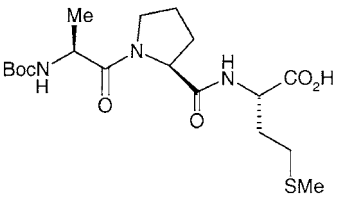
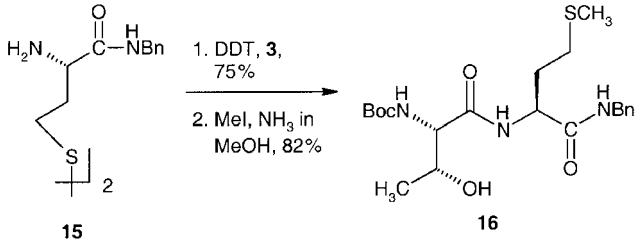
Thioesters	Ligation reaction		Methylation reaction	
	Time (h)	Yield (%)	Products[e]	Yield (%)
 **1**	5[a]	qu[b]	 **8**	84[b]
 **2**	.5	qu[c]	 **9**	76[c]
 **3**	6	96[b]	 **10**	86[b]
 **4**	24	qu[b]	 **11**	79[b]
 **5**	5	88[d]	 **12**	77[d]

Table 1 Reaction of Homocysteine with Aminoacids and Peptide Thioesters Followed by S-Methylation (continued)

Thioesters	Ligation reaction		Methylation reaction	
	Time (h)	Yield (%)	Products[e]	Yield (%)
 **6**	30	89[b]	 **13**	79[b]
 **7**	6	91[d]	 **14**	79[d]

[a] 1.25 equiv of thioester was employed.
[b] Obtained by recrystallization in petroleum ether–diethyl ether.
[c] Obtained by column chromatography (MeOH–$CHCl_3$, 9:1).
[d] Obtained by recrystallization in ethyl acetate–petroleum ether.
[e] For physical data and comparison with literature data see ref.[11]

Also, reaction of thioesters **3**–**7** with homocysteine followed by S-methylation yielded the corresponding methionine containing peptides **10**–**14** in very good yields (Table 1).[10,11] Further, as found in previous cysteine alkylation studies of unprotected peptides,[12,13] there was no overmethylation of amines, alcohols, amides, and carboxylate groups observed during methylation of the thiol group. So both the ligation and the methylation step are chemoselective and also racemisation was not observed. Hence, this method seems to be an alternative or ideally complement the cysteine based native chemical ligation. This is also confirmed by the transformation of the *N*-benzyl amide of homocysteine which is readily generated as intermediate by reductive cleavage of homocysteine derivative **15** (Scheme 2); addition of the thioester of threonine (**3**) led directly to the desired dipeptide amide intermediate which on S-methylation afforded BocThrMetNHBn dipeptide **16** in good yield. Thus, also amide bonds of the homocysteine intermediate are stable under the reaction conditions.



15 **16**

Scheme 2 Synthesis of BocThrMetNHBn (**16**)

In conclusion, the two-step synthesis of methionine containing peptides is convenient and very efficient. It is noteworthy that in the ligation step no byproducts were observed and S-methylation of the thiol homocysteine side chain with methyl iodide is a highly chemoselective process which, besides cysteine, is compatible with all side chains of the proteinogenic aminoacids. Hence, this method provides various possibilities for peptide and protein modification. Therefore, general application of this approach to the synthesis of peptides and particularly of glycopeptides is in progress in our laboratory.

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(10) **General Procedure for the Synthesis of Peptides 8–14, 16:** To a stirred solution of homocystine (200 mg, 0.75 mmol) in 0.1 M Tris buffer (10 mL, pH ca. 8.0) containing 6 M guanidinium hydrochloride was added DTT (230 mg, 1.5 mmol) and the reaction mixture was allowed to stir at r.t. After 1 h, thioester (1.5 mmol) was added to the reaction mixture. After completion of the reaction (TLC monitoring), the reaction mixture was washed with $CHCl_3$ and the aq layer was neutralized with dilute HCl; the aq layer was then extracted with EtOAc followed by washing the EtOAc layer with brine and the organic layer was dried over anhyd Na_2SO_4. Evaporation of the solvent yielded the corresponding crude thiol, which was purified by recrystallization. This thiol was immediately taken up in NH_3 in MeOH (3 mL) and MeI (3 equiv) was added into the reaction mixture at 0 °C. This reaction mixture was allowed to stir at 0 °C for 2.0 h and at r.t. for 30 min. Then the reaction mixture was neutralized with dilute HCl followed by extraction with EtOAc, washing the EtOAc layer with H_2O and the organic layer was dried over anhyd Na_2SO_4. Evaporation of the organic solvent yielded the crude product which was purified by recrystallization or column chromatography. All products were characterized by NMR data.
(11) Compounds **8**–**14**, **16** were characterized by $[\alpha]_D$, ^{1}H NMR and ^{13}C NMR data. **8**: Mp 56–60 °C (lit.[14]: 59–62 °C); $[\alpha]_D^{20}$ –27.7 (*c* 1, MeOH). **9**: Mp 82–88 °C; $[\alpha]_D^{20}$ +27.8 (*c* 1, MeOH). **10**: Mp 50–54 °C; $[\alpha]_D^{20}$ –15.3 °C (*c* 1, MeOH). **11**: Mp 70–80 °C; $[\alpha]_D^{20}$ –2.2 (*c* 1, MeOH). **12**: Mp 145–149 °C (lit.[15]: 149–154 °C); $[\alpha]_D^{20}$ –19.0 (*c* 1, DMF) (lit.15 $[\alpha]_D$ –20 (*c* 1, DMF). **13**: Mp 83–88 °C (lit.[16]: 90 °C); $[\alpha]_D$ –3.5 (*c* 1, MeOH). **14**: Mp 65 °C (petroleum ether/ethyl acetate); $[\alpha]_D^{20}$ –62.0 (*c* 1, MeOH). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.36 (d, *J* = 6.5 Hz, 3 H), 1.43 (s, 9 H), 1.99–2.30 (m, 6 H), 2.05 (s, 3 H), 2.46 (t, 6.4 Hz, 2 H), 2.90–3.10 (m, 2 H), 3.50–4.60 (m, 4 H), 6.20–6.40 (br s, 1 H), 7.40–7.60 (br s, 1 H). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 15.4, 18.1, 25.0, 28.4, 30.8, 40.8, 47.5, 48.0, 54.4, 60.8, 77.2, 79.6, 155.5, 171.5, 173.8, 177.8; MALDI: *m/z* = 456 [M + K^+]. **16**: Mp 52–56 °C; $[\alpha]_D^{20}$ –39.5 (*c* 1, MeOH). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.12 (d, *J* = 6.4 Hz, 3 H), 1.40 (s, 9 H), 1.90–2.2 (m, 2 H), 2.03 (s, 3 H), 2.50 (t, *J* = 7.4 Hz, 2 H), 3.33 (d, *J* = 3.0 Hz, 1 H), 4.06–4.09 (m, 1 H), 4.23–4.26 (m, 1 H), 4.39 (t, *J* = 5.4 Hz, 2 H), 4.56–4.65 (m, 1 H), 5.50 (d, *J* = 7.6 Hz, 1 H), 6.9 (br s, 1 H), 7.2–7.4 (m, 6 H). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 15.2, 18.5, 28.2, 30.1, 31.2, 43.5, 52.6, 58.7, 67.2, 80.4, 127.4, 127.6, 128.6, 137.8, 156.2, 170.9. 171.2. MALDI: *m/z* = 462 [M + Na^+].
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