ORGANIC LETTERS

2007
Vol. 9, No. 4
687−690

Selective Desulfurization of Cysteine in the Presence of Cys(Acm) in Polypeptides Obtained by Native Chemical Ligation

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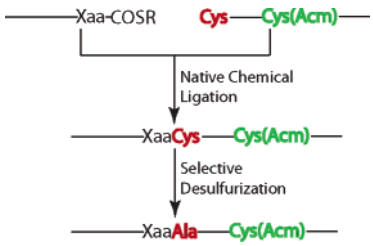
Received December 13, 2006

ABSTRACT



Increased versatility for the synthesis of proteins and peptides by native chemical ligation requires the ability to ligate at positions other than Cys. Here, we report that Raney nickel can be used under standard conditions for the selective desulfurization of Cys in the presence of Cys(Acm). This simple and practical tactic enables the more common Xaa-Ala junctions to be used as ligation sites for the chemical synthesis of Cys-containing peptides and proteins.

The most effective way to synthesize large peptides and proteins involves the use of native chemical ligation—the chemo- and regioselective reaction of a peptide-thioester and a Cys-peptide, with both peptides in side chain unprotected form, resulting in a single product with a native amide bond at the ligation site.[1] The development of a synthetic strategy for the preparation of a protein by means of native chemical ligation relies on the location of suitable Xaa-Cys ligation sites, spaced at intervals no greater than ~40 residues throughout the target amino acid sequence. However, Xaa-Cys sites in a protein's polypeptide chain are often limiting: Cys residues are rare or even absent in many proteins and when present may often be in unusable positions (e.g., not suitably spaced throughout the target sequence). For this reason, considerable efforts have been devoted to developing chemistry for amide-forming ligations at sites other than Xaa-Cys.[2]

A useful way to extend the utility of native chemical ligation to peptides and proteins without Cys residues was recently introduced by Yan and Dawson.[3] In their approach, native chemical ligation is done at Xaa-Ala sites, with a Cys residue used in place of the native Ala residue. Subsequent desulfurization of the ligation product with freshly prepared Raney nickel is used to regenerate the native Ala residue at the site(s) of ligation.[3] The method of Yan and Dawson has proven to be a very reliable and useful one.[4] We have used this native chemical ligation−global desulfurization approach to synthesize a number of sulfur-free proteins with great success.[5] In all cases, we have obtained excellent yields of the target sulfur-free polypeptide chains.

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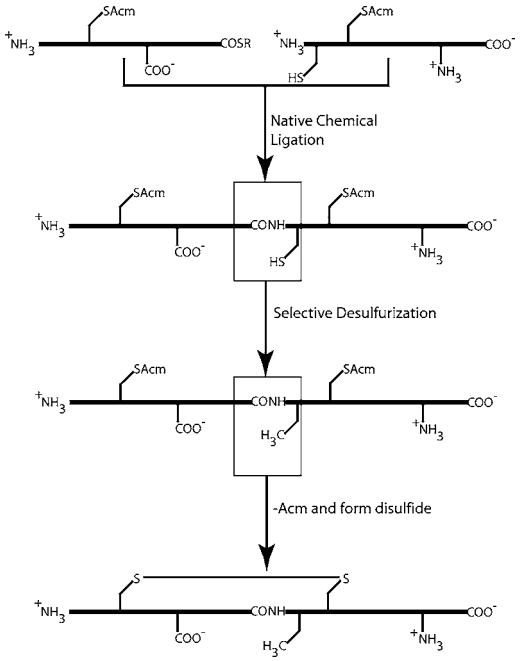
10.1021/ol0630144 CCC: $37.00
Published on Web 01/18/2007

Because of its great practical utility, we wanted to extend the use of native chemical ligation followed by desulfurization to a more versatile synthesis of Cys-containing peptide and protein targets. We set out to develop a set of chemistries that would allow native chemical ligation followed by selective desulfurization of Xaa-Cys to Xaa-Ala in the presence of side chain protected Cys (Scheme 1). This is an important

Scheme 1. General Synthetic Strategy for Cys-Containing Targets Using Native Chemical Ligation Combined with Selective Desulfurization[a]



[a] COSR represents an alkylthioester moiety.

current objective for chemical protein synthesis.[6] Although it has been generally assumed that selective desulfurization of Cys in the presence of side chain protected Cys is not possible,[3−4,7] it has been reported that under the appropriate conditions organic thiols can be reduced in the presence of thioethers.[8] We chose to focus our efforts on the $-CH_2NH-COCH_3$ (acetamidomethyl, Acm) side chain protecting group for Cys because it is amenable to Boc chemistry and is compatible with ligation chemistry, and conditions for Acm protecting group removal are well established.[9] Furthermore, we anticipated that under the right conditions Cys could be desulfurized in the presence of Cys(Acm), as suggested in work published by Hilvert et al. in their studies of selenoCys reduction.[10] Here, we report a set of chemical tactics that enable the synthesis of Cys-containing peptides and proteins by means of native chemical ligation, at sites that will become Xaa-Ala, combined with selective desulfurization.

First, we did model studies with a small peptide to test if it was possible to selectively desulfurize Cys in the presence of Cys(Acm). We found that Cys residues can in fact be selectively desulfurized in the presence of Cys(Acm), in a practical and straightforward manner. Then, we combined native chemical ligation and selective desulfurization for the total chemical synthesis of the peptide hormone amylin[11] and the small protein EETI-II.[12] To our knowledge, this is the first time that the selective desulfurization of Cys-containing peptides in the presence of Cys(Acm) has been used in the practical synthesis of peptides and proteins.

The peptide LYRACys(Acm)FGCKI (Figure 1) was prepared in a stepwise fashion using Boc chemistry solid-phase synthesis. It was subjected to reduction using freshly

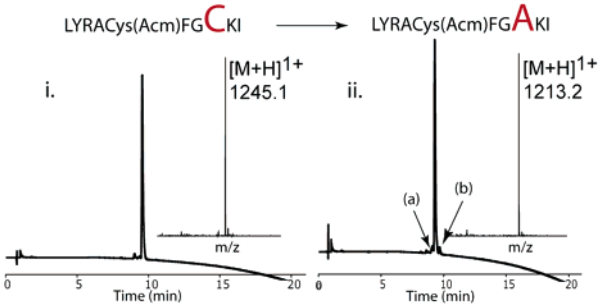


Figure 1. Model study for the selective desulfurization of Cys to Ala in the presence of Cys(Acm). Analytical reverse-phase HPLC chromatograms of: (i) starting peptide; (ii) crude products after treatment with Raney nickel. The peak labeled (a) contains desulfurized Cys(Acm), and the peak labeled (b) is starting material. The chromatographic separations were done on a Vydac C4 2.1 × 100 mm column using a linear gradient of 5−65% buffer B over 15 min at 40 °C with a flow rate of 0.5 mL/min (buffer A = 0.1% TFA in H_2O; buffer B = 0.08% TFA in acetonitrile). These conditions were used in all subsequent analyses. The eluent was monitored at 214 nm, with online electrospray MS (insets). The observed and calculated (using average isotopes) masses for each principal component were: (i) observed (obsd) = 1244.1 ± 0.5 Da, calculated (calcd) = 1244.5 Da; (ii) obsd = 1212.2 ± 0.5 Da, calcd = 1212.5 Da.

prepared Raney nickel, under the following conditions: 0.2 M aqueous phosphate, 6 M guanindine·HCl, pH 4, room temperature, 20 mM triscarboxyethlyphosphine·HCl (TCEP).

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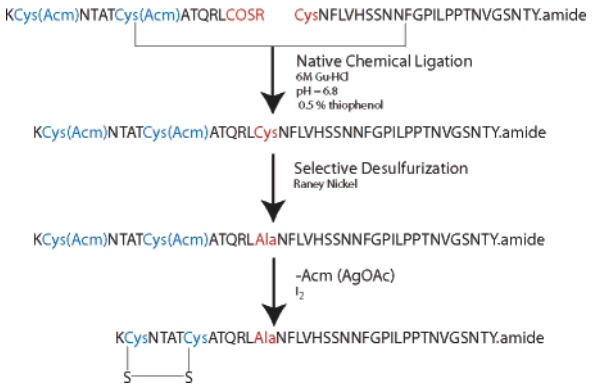
Scheme 2. Synthetic Strategy Used for the Synthesis of [A24P,S27P,S28P]amylin[a]



[a] COSR = alkylthioester.

Cys was converted to Ala quantitatively in less than 2 h as determined by LCMS. Only trace amounts of desulfurization of Cys(Acm) were observed (Figure 1).

Next, we explored the synthesis of a 37-residue peptide hormone, [A24P,S27P,S28P]amylin; the three Pro substitutions have been shown to minimize aggregation.[13] Amylin has a C-terminal caboxamide and has one disulfide bond between Cys2 and Cys7 and is thus a suitable biologically active peptide in which to illustrate selective reduction of Cys in the presence of Cys(Acm). The synthetic strategy for the total chemical synthesis of this target peptide is shown in Scheme 2.

Figure 2 shows the data for the synthesis of the [A24P,-S27P,S28P]amylin polypeptide. Native chemical ligation of the two peptides amylin[1-12]-$^{\alpha}$thioester and [Cys^{13}-37]amylin gave the full-length polypeptide in high yield. The purified ligation product that contained a single Cys residue in the presence of two Cys(Acm) residues was subjected to Raney nickel reduction. After 6 h, the reduction was complete and the product appeared as a single symmetrical peak with a mass decrease of 32.1 Da. Solid-phase extraction (SPE) was used to isolate the desulfurized product, which was eluted with 0.1% TFA in 50:50 acetonitrile/water. The Cys(Acm) protecting groups were removed, and the disulfide bond was formed by oxidation with iodine,[14] giving a high yield of [A24P,-S27P,S28P]amylin (see Supporting Information for details).

Next, we undertook the total chemical synthesis of the protein EETI-II using native chemical ligation and selective desulfurization. EETI-II is a small trypsin inhibitor protein that contains three disulfide bonds.[12] The synthetic route for [Met7Nle]EETI-II[1-28] involves the selective desulfurization of residue Cys17 to give Ala17, in the presence of six Cys(Acm) residues, after the ligation of the EETI-II peptide segments [1-16]-$^{\alpha}$thioester and Cys17-28 (Scheme 3). Thus,

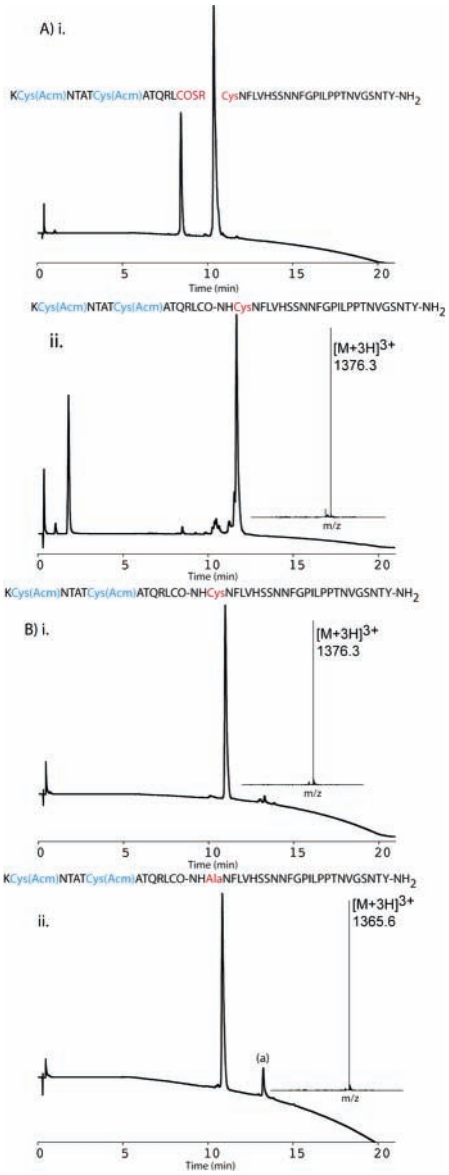


Figure 2. Analytical HPLC data for the synthesis of [A24P,S27P,-S28P]amylin. Insets: Electrospray MS data for the principal component. (A) (i) time = 0 h for the ligation reaction; (ii) time = 8 h for reaction completion. The observed and calculated masses for the product: obsd = 4125.9 ± 0.5 Da, calcd = 4125.6 Da. (B) Data for the selective desulfurization of amylin: (i) time = 0 for the reaction; (ii) time = 6 h, crude products showing complete desulfurization (peak (a) is an unrelated column contaminant). See Supporting Information for details of HPLC configurations used. Observed and calculated masses at each step were: (i) obsd = 4125.9 ± 0.5 Da, calcd = 4125.6 Da; (ii) obsd = 4093.8 ± 0.5 Da, calcd = 4093.6 Da.

this system is an excellent model to demonstrate the exquisite selectivity of the desulfurization reaction. Subsequently, the acetamidomethyls were removed and the product folded to form three disulfide bonds (see Supporting Information). The analytical data for the synthesis of the EETI-II polypeptide chain by native chemical ligation followed by selective desulfurization is shown in Figure 3. The ligation between

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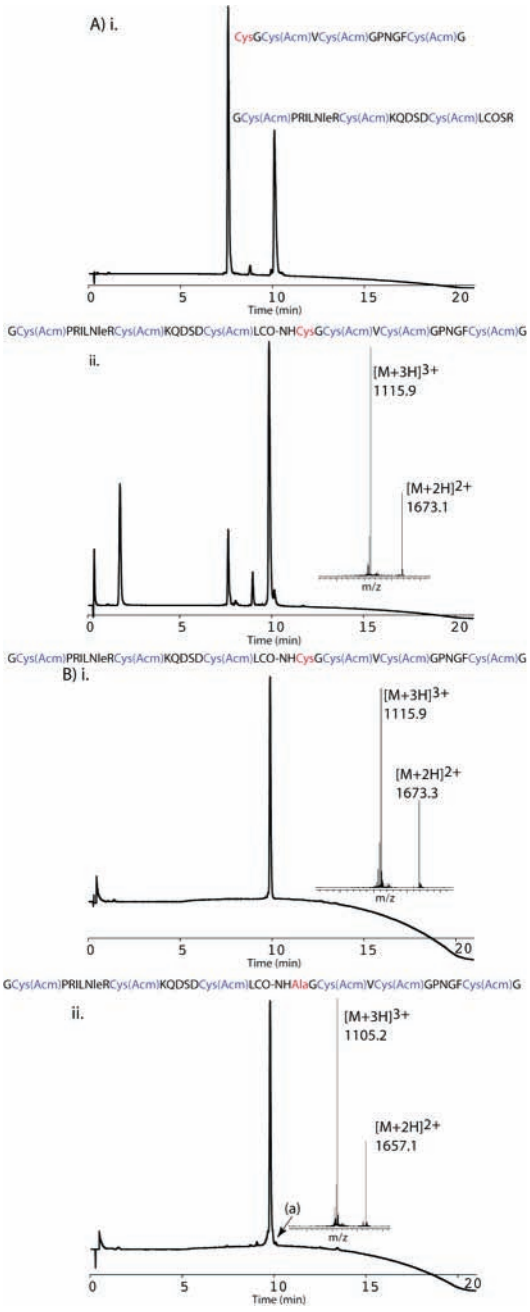
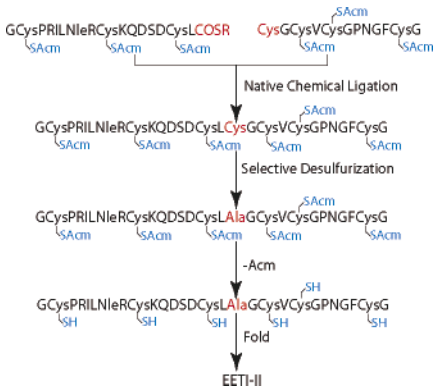


Figure 3. Analytical HPLC data for the synthesis of [Met7Nle]-EETI-II[1-28]. (A) Ligation reaction: (i) time = 0; (ii) time = 8 h crude products. Inset: Electrospray MS data for the principal product. Observed and calculated masses were: obsd = 3344.5 ± 0.5 Da, calcd = 3345.0 Da. (B) Selective desulfurization: (i) time = 0; (ii) time = 6 h 30 min; crude products, peak (a) is residual starting material. Insets: Electrospray MS data for the principal components. Observed and calculated masses at each step were: (i) obsd = 3344.5 ± 0.5 Da, calcd = 3345.0 Da; (ii) obsd = 3312.6 ± 0.4 Da, calcd = 3312.9 Da.

EETI-II[1-16]-$^{\alpha}$thioester and [Cys17-28]EETI-II took approximately 8 h and resulted in a 58% yield.

The purified ligation product was then desulfurized and isolated in 89% yield. The six Acm groups were removed with iodine, after which the kinetically formed disulfides were reduced with DTT and the protein folded with the formation of native disulfides using a glutathione redox couple. The folding reaction was monitored with LCMS, and the mass of the oxidized product decreased by 6 Da, corresponding to the formation of three disulfides. The folded protein eluted earlier on reverse-phase HPLC as previously reported (see Supporting Information).[15]

Scheme 3. Synthetic Strategy Used for the Total Synthesis of EETI-II[1-28]



In other studies, we investigated the selective reduction of Cys in the presence of Met. We found that Cys can be quantitatively desulfurized to Ala in the presence of Met and Cys(Acm) using the mild conditions reported here (see Supporting Information). This extends the original observations of Yan and Dawson.[3]

Efficient and selective desulfurization of Cys in the presence of Cys(Acm) is feasible as demonstrated by syntheses of the peptide hormone amylin and the small model protein EETI-II. With selective desulfurization, proteins can be built by native chemical ligation from disconnections at the more common Xaa-Ala ligation sites, even in peptide and protein targets that contain other Cys residues. Furthermore, as pointed out by Yan and Dawson, native chemical ligation can be used with any amino acid that has been modified to have a thiol moiety on the β-carbon, e.g., [β-SH]Phe.[3] Subsequent desulfurization regenerates the naturally occurring amino acid at the ligation site. In this way, a variety of Xaa-Yaa sequences can be used as ligation sites. Native chemical ligation in combination with selective desulfurization for the total synthesis of Cys-containing polypeptides thus represents a very useful expansion of the scope of the chemistries for the total synthesis of large peptides and proteins.

Acknowledgment. This research was supported by the Department of Energy Genomes to Life Genomics Program (Grant DE-FG02-04ER63786 to S.B.K.).

Supporting Information Available: Experimental details for peptide synthesis, native chemical ligation, selective desulfurization, and EETI-II folding. This material is available free of charge via the Internet at http://pubs.acs.org.

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