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A route to cyclic peptides and glycopeptides by native chemical ligation using in situ derived thioesters

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Abstract—The synthesis of cyclic peptides and glycopeptides by native chemical ligation using in situ derived thioesters is described.

We have initiated a program seeking to enable the full chemical synthesis of glycopolypeptides and ultimately glycoproteins. We were initially drawn to this problem by the intellectual and experimental challenges of developing a workable chemical estate that would accommodate the multifaceted nuances of two of the three major classes of biomolecular building blocks. Moreover, there were specific target structures (i.e., isoforms of prostate specific antigen[1] as well as candidate antigens around which to generate gp120-directed HIV vaccines[2]), which served to focus our efforts. A central premise of our enterprise is that Nature's elaborate program to achieve post-translational glycosidation of proteins is presumably driven by important advantages accruing from the elaboration of saccharide domains in proteins.[3] Therefore, access to homogeneous complex glycopolypeptides could be of considerable value in understanding the biology-level consequences of protein glycosidation.

We had initiated our program by learning how to build N-linked glycopeptides.[4] In these early studies, we focused on constructing fully synthetic complex oligosaccharides and attaching them to small peptide domains through adaptation of the Kochetkov–Lansbury anomeric amination–aspartylation methodology[5] to total synthesis contexts. In the next phase, we learned how to ligate fully synthetic oligosaccharide-small peptide domains using (cysteine-based) native chemical ligation.[6] These methodologies were then applied to the total synthesis of prostate specific antigen glycoforms,[1] as well as to the carbohydrate domain of gp120 linked to a small polypeptide domain.[2]

More recently we have described a new method to generate, in situ, an active thioester containing a fully synthetic oligosaccharide domain (Scheme 1).[7] The incipient acyl donor can then be coupled, through native chemical ligation, to a glycopolypeptide through its N-terminal cysteine moiety. There was thus provided a totally synthetic polypeptide with two N-linked oligosaccharide domains.

In their linear form, biologically relevant peptides can assume a baffling number of conformations. However, it is likely that only select conformations bind to their receptor with high affinity. Conformational constraints are often applied in order to evaluate the structural and dynamic properties that are important to the selectivity and potency of peptides. Surely one of the most informational modifications that can impart significant amounts of conformational rigidity is brought about by cyclization. Through ring formation, one has the ability to systematically study the biologically active conformations and perhaps infer their significance in the linear peptides. Moreover, cyclic peptides have many advantages over their open-chain counterparts, including increased bioavailability, metabolic stability and receptor selectivity.[8] Accordingly, cyclic peptides have become a common starting point in drug development.[9]

Tam et al. has demonstrated the applicability of native chemical ligation to the synthesis of cyclic peptides.[10,11]

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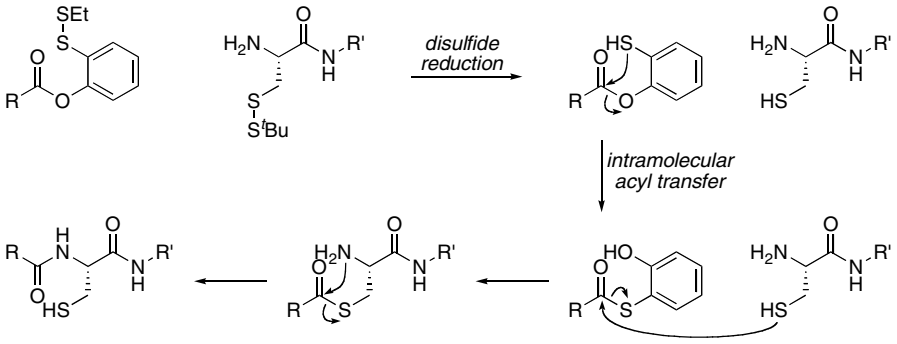


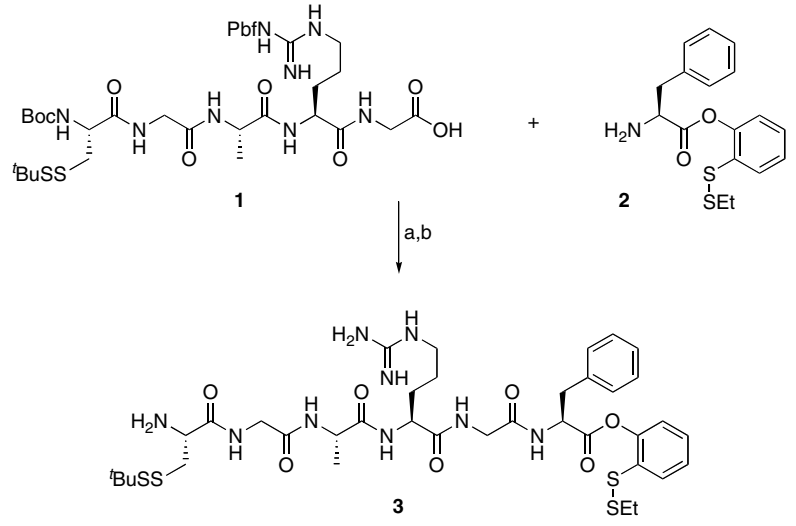
Scheme 1. Presumed mechanism of in situ thioester generation and subsequent ligation.

In this case, the linear peptides of interest were generated using standard Boc (*tert*-butyloxycarbonyl)-based solid phase synthesis, and were obtained as discrete peptide alkyl thioesters. Cyclization then proceeded at pH $\geqslant$ 6.4 in phosphate buffer containing a 3–5-fold excess of 3-mercaptopropionic acid. However, given the expected incompatibility of many glycosidic linkages to Boc-based SPPS,[12] we initially set out to determine if our proposed method could be extended to provide a simple entry into this class of compound. Thus, peptide acid **1** was prepared using standard Fmoc-based SPPS and then converted into the cyclization precursor **3**, by direct acylation with **2** followed by acidic removal of the Boc and Pbf protecting groups (Scheme 2).

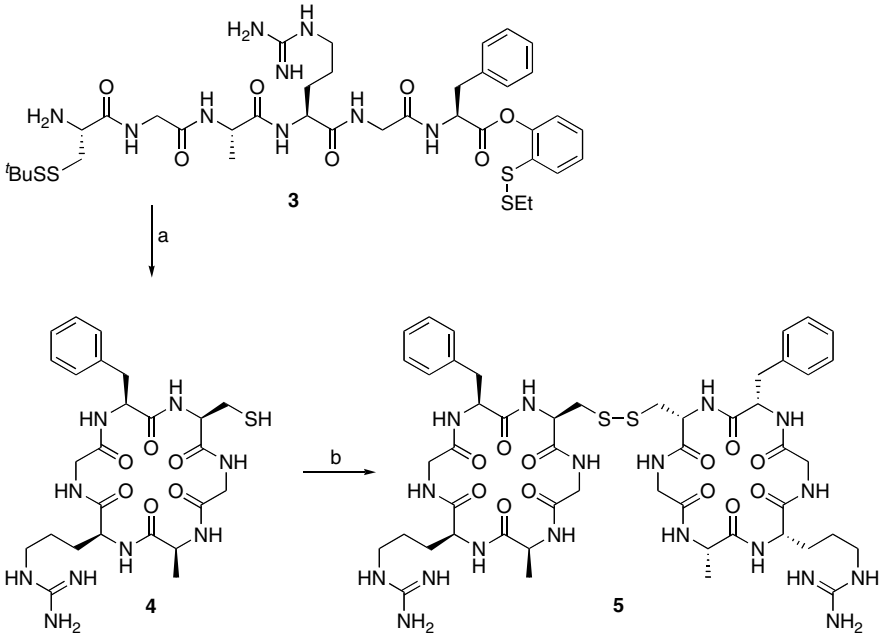
With **3** in hand, we investigated the applicability of various cyclization conditions. In the event, treatment of **3** with 2-mercaptoethanesulfonic acid sodium salt (MesNa) in phosphate buffered saline (pH 7.4) afforded cyclic peptide **4** in an encouraging 78% yield. The reaction itself occurred quite rapidly, with complete consumption of the starting material within 3 h. LCMS analysis during and after the reaction gave no indication of thioester hydrolysis. Moreover, we were quite excited to find that there were no oligomerized products detected in the reaction mixture. This possibility had been of concern since both ends of the linear peptide precursor are highly reactive and head to tail cyclization of peptides containing less than seven residues is often problematic.[13] The cyclic nature of the peptide was confirmed by formation of disulfide **5** upon exposure of **3** to hydroxylamine. No trace of products arising from hydroxylamine addition was observed by LCMS analysis of the disulfide[14] (Scheme 3).

The pH dependence on the rate and yield of the reaction was examined at three discrete values (pH 4.5, 6.0 and 7.4). Tam has reported that, at low pH, the concentration of anionic thiolate is not high enough to compete with hydrolysis of the thioester.[15] We found this to be the case in our system as well. The reaction was slowest and accompanied by the highest amount of hydrolysis at pH 4.5, although starting material was still present even after stirring for 15 h at room temperature. At pH 6.0, the reaction occurred much faster (completion after 7 h), however a small amount of the hydrolysis product was still observed. At pH 7.4, the reaction proceeded so rapidly that virtually no hydrolysis occurred.

Covalently bound oligosaccharides can significantly affect the biophysical properties displayed by a given protein.[16] Indeed, the clinical relevancy of the pleiotro-



Scheme 2. Reagents and conditions: (a) EDCI, HOBt, DMF, CH_2Cl_2; (b) TFA, H_2O, PhOH (67% over two steps). EDCI = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, HOBt = 1-hydroxybenzotriazole, DMF = dimethylformamide, TFA = trifluoroacetic acid.

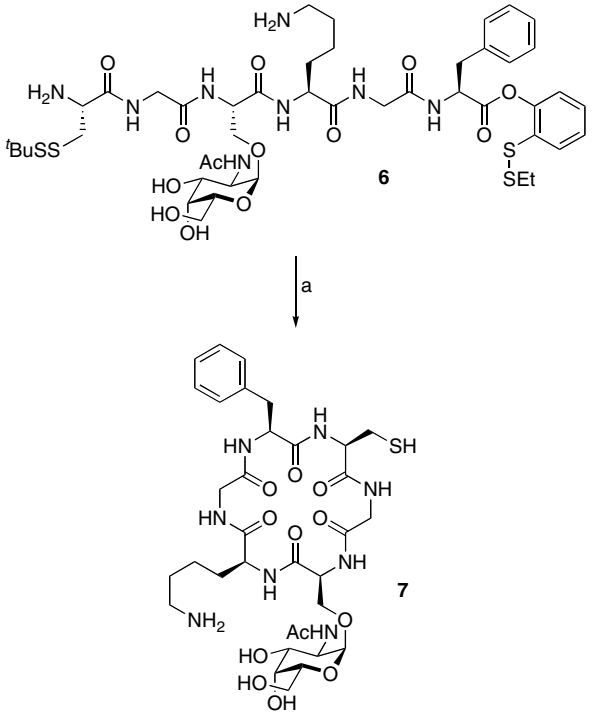


Scheme 3. Reagents and conditions: (a) MesNa, PBS (pH = 7.4), then TCEP (78%); (b) NH_2OH, H_2O. MesNa = 2-mercaptoethane sulfonic acid, TCEP = tris(carboxyethyl)phosphine, PBS = phosphate buffered saline.

phic cytokine, erythropoietin is amplified upon glycosylation.[17] Furthermore, the mannopeptimycin family of antibiotics is an important emerging class of cyclic glycopeptides active against vancomycin-resistant bacteria.[18] Importantly, it is believed that the carbohydrate domain of these compounds is responsible for their biological activity.[19] Glycosylation of in place cyclic peptides is not considered a viable option for the generation of such compounds, as the peptidic substrates are often insoluble under the conditions typically used for successful glycosidation.[20] Having demonstrated the applicability of our methodology to the synthesis of cyclic peptides, we felt it appropriate to extend the scope of the reaction to include cyclic glycopeptides. In doing so, we minimize solubility concerns since the carbohydrate is already attached to the peptide prior to cyclization. Moreover, issues such as microheterogeneity in the glycodomain are avoided because well-tested methodologies can be employed in the synthesis of the linear glycopeptide.[21]

Thus, glycopeptide **6**, containing a single O-linked galactosamine moiety was synthesized using a procedure similar to that of **1**. The carbohydrate was incorporated using the cassette method for glycopeptide synthesis.[21] As before, smooth cyclization was observed following treatment of the glycopeptide with MesNa in PBS (pH 7.4), providing cyclic glycopeptide **7**. Again, the reaction proceeded rapidly with complete consumption of the starting material in 3 h (Scheme 4).

With these milestones accomplished, the program to reach homogeneous glycopolypeptides and even complex glycoproteins moves to encompass more challenging systems.



Scheme 4. Reagents and conditions: (a) MesNa, PBS (pH = 7.4) then TCEP (73%).

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