Chemoselective backbone cyclization of unprotected peptides

Julio A. Camarero and Tom W. Muir*

Laboratory of Synthetic Protein Chemistry, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

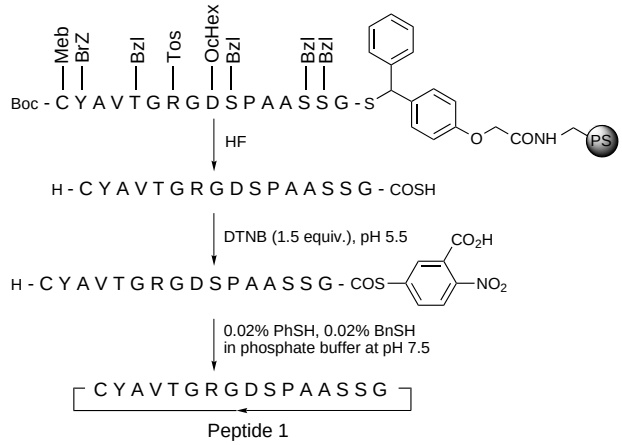
A fully unprotected 15 residue peptide containing an N-terminal Cys residue and a C-terminal thioester moiety is cleanly converted to the corresponding head-to-tail cyclic peptide in aqueous buffers at around neutral pH.

Cyclic peptides have restricted conformational flexibility compared to their linear counterparts, a feature which makes them of great interest in studying the structure–activity relationships of bioactive peptides.[1,2,3] Classical solution-based approaches to the synthesis of amide-cyclized peptides rely on fully protected linear precursors which are selectively activated and cyclized under conditions of high dilution.[4] Although this methodology has been used with satisfactory results in the synthesis of small cyclic peptides,[5] its application to the cyclization of larger peptides is constrained by the decreasing solubility of fully protected peptides as their size increases. To overcome these solubility problems, many researchers have utilized a variety of minimal protection strategies.[6] However, cyclization of minimally protected fragments *via* conventional carboxylate activation chemistries can lead to the accumulation of unwanted side-products, resulting in the desired material being generated in only moderate yield.[6]

In a recent report, Tam and co-workers describe a powerful new route to cyclic peptides in which unprotected peptide precursors are chemoselectively cyclized in aqueous solution.[7] This approach utilizes a thiazolidine-forming[8] intramolecular chemical ligation reaction, and was used to generate a series of cyclic peptides in good yield from soluble linear peptide precursors.[7] Here we describe a complementary methodology which facilitates the rapid and quantitative backbone cyclization of fully unprotected peptides in aqueous solution. Our cyclization strategy is based on an intramolecular native chemical ligation reaction,[9] and therefore requires the presence of an N-terminal Cys† residue and a C-terminal thioester moiety within the linear peptide precursor. We have successfully applied this new cyclization approach to the generation of backbone cyclized model peptide **1**, the sequence of which is derived from the tenth type 3 module of Fibronectin, and contains the RGD motif known to be involved in binding to the integrin class of cell adhesion receptor.[10]

The synthetic strategy used to generate cyclic peptide **1** is illustrated in Scheme 1. The fully protected linear precursor was assembled manually by stepwise SPPS using the *in situ* neutralization/HBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] activation protocol for Boc chemistry.[11] The synthesis was carried out on a Boc-Gly-(thioester handle)-aminomethyl-polystyrene resin[12,13] on a 0.1 mmol scale. After the synthesis was complete, the cleavage and total deprotection was accomplished by treatment with anhydrous HF–*p*-cresol (95 : 5, 10 ml, 0 °C for 1 h). Following trituration with cold diethyl ether, the crude peptide-$^{\alpha}$thioacid was extracted with 0.1% TFA in MeCN–H_2O (1 : 1, 15–20 ml) and lyophilised. Analysis of the crude material by analytical C18 reverse-phase HPLC (10–40% B‡ over 30 min) gave a major peak whose mass was consistent with that of the expected peptide-$^{\alpha}$COSH [calculated molecular weight (av. isotope comp.) = 1444.5 Da; found = 1443.9 ± 0.1 Da]. In order to transform the $^{\alpha}$thioacid function into an active $^{\alpha}$thioester, the lyophilised crude material (65 mg, 45 μmol) was dissolved in a buffer containing 6 M GuHCl, 0.1 M sodium phosphate, pH 5.5 (30 ml), and a solution of DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] in 0.1 M phosphate buffer, pH 5.5 (15 ml of a 5 mM solution, 1.5 equiv.) was added at room temperature. The thioesterification reaction was complete after 30 min,§ and the major product was characterized by ESMS (electrospray mass spectrometry) as being the desired peptide-$^{\alpha}$thioester [calculated molecular weight (av. isotope comp.) = 1607.6; found = 1605.9 ± 0.1 Da]. Upon completion, the reaction was quenched by the addition of 0.1% TFA in H_2O, and the peptide-$^{\alpha}$thioester product purified by preparative HPLC (C18 reverse-phase HPLC column, 18–23% B over 45 min at 50 ml min^{-1}) in high purity [10 mg, 6.2 μmol, Fig. 1(*a*)]. It is worth noting that the lower pK_a of the $^{\alpha}$thioacid group relative to the cysteine sulfhydryl group ensures that the predominant reaction with DTNB at pH 5.5 is thioesterification and not disulfide formation.[14]

Cyclization of the linear peptide precursor (containing the necessary N-terminal Cys and C-terminal thioester group) to give peptide **1** was accomplished under high dilution conditions as follows. The purified peptide-$^{\alpha}$thioester was dissolved to a final concentration of *ca*. 50 μM in a freshly degassed buffer (pH 7.5) containing 0.1 M sodium phosphate, 1 mM EDTA, and both PhSH and BnSH at a concentration of 0.02% v/v.¶ In order to monitor the course of the reaction, small aliquots (100 μl) of the reaction mixture were periodically removed, quickly quenched by addition of 10% TFA in H_2O (20 μl), and then analysed by analytical C18 reverse-phase HPLC. Under these conditions, the backbone cyclization reaction was found to be completely chemoselective, since only the desired cyclomonomeric peptide **1** was obtained [Fig. 1(*b*)]. Moreover, cyclization proceeded extremely fast and the linear starting material was cleanly converted to the cyclic product within 10 min [Fig. 1(*c*)]. Cyclic peptide **1** was purified by semipreparative C18 reverse-phase HPLC (10–40% B over 45 min at ml min^{-1}) and characterized by ESMS [calculated molecular weight (av.



Scheme 1

isotope comp.) = 1408.5 Da; found = 1408.4 ± 0.6 Da] and tryptic digestion.‖

In summary, we have described a practical new strategy which allows fully unprotected peptides to be cyclized in a head-to-tail manner in aqueous solution at neutral pH. In the example described, cyclization was observed to be both clean and rapid, and afforded the desired cyclic peptide **1** in excellent yield. As with intermolecular native chemical ligation,[9] this intramolecular cyclization reaction can be performed in the presence of all naturally occurring amino acid side-chains, including additional cysteine sulfhydryls. The compatibility of this cyclization chemistry with native protein folding conditions coupled with recent advances in the total chemical synthesis of proteins,[15] suggests a potentially straightforward route to circular versions of small proteins and protein domains.

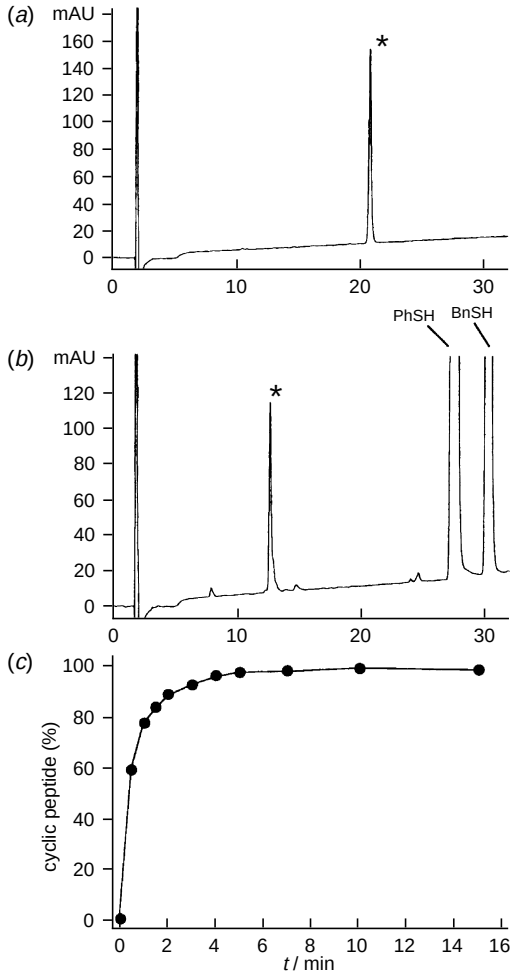


Fig. 1 Reverse-phase HPLC analysis of peptide **1** cyclization. (*a*) peptide-$^{\alpha}$COSNB after HPLC purification. (*b*) cyclization reaction after 15 min. (*c*) progress of the cyclization reaction. The desired product in each panel is indicated by an asterisk.

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Footnotes

* E-mail: muirt@rockvax.rockefeller.edu

† A more general approach to native (amide-forming) chemical ligation that extends the technique beyond the originally reported X-Cys ligation site to X-Gly and Gly-X ligation sites has been described recently.[16]

‡ Where A = 0.1% TFA in H_2O and B = 0.1% TFA in MeCN : H_2O (9 : 1 v/v).

§ Thioesterification of peptide-$^{\alpha}$thioacids with aryl disulfides such as DTNB goes *via* an acyl disulfide intermediate.[17]

¶ Inclusion of BnSH and PhSH in the ligation buffer results in the *in situ* conversion of the 2-nitrobenzoic peptide-$^{\alpha}$thioester (peptide-$^{\alpha}$COSNB) into the corresponding benzyl and phenyl peptide-$^{\alpha}$thioesters through trans-thioesterification.[18] The presence of these aromatic thio cofactors was found to eliminate unwanted peptide-$^{\alpha}$thioester hydrolysis.

‖ Trypsin hydrolysis in 0.1 M Tris HCl buffer pH 8.5 at the unique Arg residue yielded only a single product, as expected for a cyclomonomeric structure, that did not co-elute with the peptide **1** and gave a molecular peak 18 Da higher than the cyclic peptide [molecular weight calculated (av. isotope comp.) = 1426.5 Da; found = 1426.4 ± 0.6 Da].

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