Published in final edited form as:

Tetrahedron Lett. 2013 August 28; 54(35): 4746–4748. doi:10.1016/j.tetlet.2013.06.118.

A Convergent Synthesis of Homogeneous Reducible Polypeptides

Mark D. Ericson and Kevin G. Rice*

Division of Medicinal & Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City IA 52242

Abstract

The convergent syntheses of homogeneous disulfide cross-linked polypeptides are reported. Reducible polypeptides were synthesized containing four and eight dodecapeptides in two and three linear conjugation steps. Critical for the convergent methodology was the use of orthogonally protected cysteines as either acetamidomethyl (Acm) or Fmoc-thiazolidine (Thz). Both groups could be selectively deprotected with silver trifluoromethanesulfonate in the presence of internal disulfide bonds using TFA and aqueous conditions, respectively. This approach allows for large, reducible polypeptides to be synthesized in efficient yields and minimizes the number of conjugation steps, allowing the development and optimization of gene delivery polypeptides containing multiple peptide components necessary to overcome the numerous *in vivo* barriers for efficacious gene delivery.

Keywords

Reducible polypeptides; convergent synthesis; thiazolidine; acetamidomethyl; cysteine

Systemic delivery of exogenous plasmid DNA *in vivo* requires successful navigation of many barriers.¹⁻³ For efficacious expression, a gene delivery peptide or polymer must protect the plasmid during circulation, selectively target and internalize within a particular tissue, escape the endosomal pathway before degradation, and translocate into the nucleus for expression. There has been much progress on the ability to overcome individual barriers: PEGylated polyacridine peptides have demonstrated the ability to protect plasmid DNA for several hours during circulation in mice,^{4,5} N-glycan derivatized peptides can target radiolabelled DNA to hepatocytes and dendritic cells,⁶⁻⁸ and fusogenic peptides such as melittin have been shown to dramatically increase gene expression *in vitro*.⁹⁻¹¹ Despite these advances, a polypeptide that incorporates all of the different component peptides has yet to produce significant levels of gene expression *in vivo* when i.v. dosed.

Critical for successful gene delivery is the assembly of the polypeptide to ensure that component peptides can function at the necessary stages of the delivery process. The binding domain must protect the DNA in serum and release the plasmid after cellular uptake, fusogenic peptides must aid endosomal escape following internalization, and nuclear localizing sequences must target after release into the cytoplasm. One method to trigger the

© 2013 Elsevier Ltd. All rights reserved.

*To Whom Correspondence Should be Addressed: Tel: 319-335-9903, Fax: 319-335-8766, kevin-rice@uiowa.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

activity of the peptides after cellular uptake is to assemble polypeptides through reducible disulfide bonds. Due to the relatively high levels of glutathione and other reducing equivalents inside a cell,¹² disulfide bonds are readily cleaved following internalization. The resulting triggered breakdown of a polypeptide provides a cellular signal to activate the component peptides. Heterogeneous reducible polypeptides have been shown to mediate higher levels of gene expression compared to non-reducible controls *in vitro*,¹³⁻¹⁵ though the resulting polypeptide mixtures from random polymerization hindered further development. Optimization of nonviral gene delivery polypeptides requires the ability to precisely control their architecture, to ascertain the ideal placement and amount of the component peptides.¹⁶

To generate homogeneous reducible polypeptides, we recently introduced a novel method of coupling peptides through disulfide bonds in a iterative reducible ligation strategy.¹⁷ Key to our method was the use of an N-terminal Fmoc-thiazolidine (Thz) that could be selectively unmasked to a free Cys in the presence of acidic aqueous silver trifluoromethanesulfonate without scrambling of previously formed disulfide bonds. While successful, the overall yield for longer polypeptides was low due to the linear synthetic approach. To limit the number of linear ligation steps, other reducible ligation strategies were evaluated for use concurrently with the Thz chemistry to create a convergent approach. Previously reported chemistries involved deprotection of acetamidomethyl (Acm) protected Cys using sulfenyl chloride derivatives¹⁸⁻²⁰ or with silver in a TFA/anisole solution.²¹⁻²³ Sulfenyl chlorides have been reported to react with the indole ring of Trp, limiting this strategy to peptides lacking aromatic amino acids.^{24,25} More intriguing was the use of silver in TFA to deprotect a Cys(Acm), representing an orthogonally protected Cys that could be selectively deprotected in the presence of disulfide bonds and Fmoc-Thz.

This communication describes the convergent synthetic methodology of reducible ligation utilizing both Fmoc-Thz and Acm protected Cys. The utility of the orthogonal protecting groups is demonstrated in the synthesis of polypeptides containing four and eight peptides subunits in two and three linear conjugation steps, and can be readily expanded into polypeptides with an even greater number of subunits.

The synthesis of polypeptide **7** was accomplished in two linear conjugation steps (Scheme 1). Donor peptide **1** with a free sulfhydryl was coupled to acceptor peptide **2** with a sulfanylpipridine in 100 mM ammonium acetate pH 5 to generate polypeptide **5**. Following preparative purification, the Fmoc was removed with 5 v/v % piperidine in DMF and the thiazolidine hydrolyzed with 50 mol eq AgOTf in 0.1 v/v % TFA as previously reported.¹⁷ The free sulfhydryl was then activated with 10 mol eq dithiodipyrindine (DTDP) to generate acceptor polypeptide **5b**. Following each step, the intermediate was purified using Sephadex G-10 resin eluted with 0.1 v/v % TFA. Similarly, donor **3** and acceptor **4** were coupled to generate polypeptide **6** containing a C-terminal Cys(Acm). The Acm was subsequently removed with AgOTf in a 96:4 TFA:anisole solution to generate the donor polypeptide **6a** which was used without further purification. Acceptor **5b** and donor **6a** were then coupled in 100 mM ammonium acetate pH 5 containing 0.5 w/v % ammonium chloride to precipitate excess silver from the generation of **6a**. Following preparative purification, polypeptide **7** was recovered as a single product in 36 % yield from purified peptides **1-4** (Fig. 1A). This represents an increase in the yield for a 4-member polypeptide generate from Thz chemistry in a linear fashion (12 %)¹⁷ and comparable to the reported convergent synthesis utilizing Acm alone (36 %).²³

The advantage to utilizing both Fmoc-Thz and Cys(Acm) is larger polypeptides can be generated, where intermediate polypeptides can be synthesized to contain both protected Cys and can be elongated from either terminal. Polypeptide **8** was assembled from donor **3** and acceptor **2** to contain both an C-terminal Cys(Acm) and N-terminal Fmoc-Thz (Scheme

2). The AcM was selectively removed using 100 mol eq AgOTf in 96:4 TFA:anisole and coupled with acceptor polypeptide **5b** to generate the four member polypeptide **9**, retaining an N-terminal Fmoc-Thz for further elongation (Fig. 1B). Conversely, the Fmoc-Thz could be selectively removed from **8**, activated as an acceptor peptide with DTDP, and conjugated with donor **6a** to generate polypeptide **10** retaining a C-terminal Cys(AcM) (Fig. 1C). While both the AcM and Thz removal rely on AgOTf, the selection of solvent proved critical for successful deprotection. The AcM was stable in aqueous AgOTf, while the Fmoc-Thz was stable in TFA containing AgOTf. A Thz without an Fmoc was hydrolyzed in TFA when AgOTf was present (unpublished results).

To generate the 8 member polypeptide **11**, polypeptide **9** was converted to the acceptor **9b** and polypeptide **10** was converted to the donor **10a** as previously described. Polypeptides **9b** and **10a** were then coupled in 100 mM ammonium acetate with 0.5 w/v % ammonium chloride, pH 5 to generate the polypeptide **11** ligated through 7 disulfide bonds, which was preparatively purified to yield a single product peak (Fig. 1D). The mass-spectral characterization of this final product proved difficult due to the large size and relatively instability of the disulfide bonds. An increased ionization energy required to ionize the 12.8 kDa polypeptide resulted in more fragmentation through fissure of the disulfide bonds. An overall yield of 4.5 % was observed in the synthesis of polypeptide **11** from purified peptide **1-4**, representing the ligation of 8 individual peptides into one polypeptide. Full mass spectral characterization and yields for all peptides and polypeptides are reported in Table 1.

In summary, we demonstrated a convergent route to large polypeptides by selectively utilizing orthogonally protected Fmoc-Thz and Cys(AcM). This improves the yields and number of peptide subunits incorporated into reducible polypeptides from previous reports and efforts to synthesize gene delivery polypeptides are ongoing. Systemically optimizing the location and amount of each component peptide may then result in polypeptides with potent gene delivery capabilities *in vivo*.

Acknowledgments

The authors gratefully acknowledge funding for this research by the NIH GM087653, T32 GM008365 and a predoctoral fellowship from the American Foundation for Pharmaceutical Education (MDE).

References

1. Fernandez CA, Rice KG. Mol. Pharmaceutics. 2009; 6:1277–1289.
2. Rettig GR, Rice KG. Expert Opin. Biol. Ther. 2007; 7:799–808. [PubMed: 17555366]
3. Wiethoff CM, Middaugh CR. J. Pharm. Sci. 2003; 92:203–217. [PubMed: 12532370]
4. Fernandez CA, Baumhover NJ, Duskey JT, Khargharia S, Kizzire K, Ericson MD, Rice KG. Gene Ther. 2011; 18:23–37. [PubMed: 20720577]
5. Kizzire K, Khargharia S, Rice KG. Gene Ther. 2013; 20:407–416. [PubMed: 22786534]
6. Collard WT, Yang YS, Kwok KY, Park Y, Rice KG. J. Pharm. Sci. 2000; 89:499–512. [PubMed: 10737911]
7. Kwok KY, Park Y, Yang YS, McKenzie DL, Liu YH, Rice KG. J. Pharm. Sci. 2003; 92:1174–1185. [PubMed: 12761807]
8. Yang YH, Park Y, Man SH, Liu YH, Rice KG. J. Pharm. Sci. 2001; 90:2010–2022. [PubMed: 11745760]
9. Baumhover NJ, Anderson K, Fernandez CA, Rice KG. Bioconjugate Chem. 2010; 21:74–83.
10. Chen C-P, Kim J.-s, Steenblock E, Liu D, Rice KG. Bioconjugate Chem. 2006; 17:1057–1062.
11. Legendre JY, Trzeciak A, Bohrmann B, Deuschle U, Kitas E, Supersaxo A. Bioconjugate Chem. 1997; 8:57–63.

12. Smith CV, Jones DP, Guenther TM, Lash LH, Lauterburg BH. *Toxicol. Appl. Pharmacol.* 1996; 140:1–12. [PubMed: 8806864]
13. McKenzie DL, Kwok KY, Rice KG. *J. Biol. Chem.* 2000; 275:9970–9977. [PubMed: 10744672]
14. McKenzie DL, Smiley E, Kwok KY, Rice KG. *Bioconjugate Chem.* 2000; 11:901–909.
15. Read ML, Bremner KH, Oupicky D, Green NK, Searle PF, Seymour LW. *J. Gene Med.* 2003; 5:232–245. [PubMed: 12666189]
16. Szoka F. *Science.* 2008; 319:578–579. [PubMed: 18239114]
17. Ericson MD, Rice KG. *Biopolymers.* 2012; 98:510–517. [PubMed: 23203756]
18. Ottl J, Battistuta R, Pieper M, Tschesche H, Bode W, Kuhn K, Moroder L. *FEBS Letters.* 1996; 398:31–36. [PubMed: 8946948]
19. Ottl J, Moroder L. *Tetrahedron Lett.* 1999; 40:1487–1490.
20. Ottl J, Moroder L. *J. Am. Chem. Soc.* 1999; 121:653–661.
21. Futaki S, Ishikawa T, Niwa M, Yagami T, Kitagawa K. *Tetrahedron Lett.* 1996; 37:201–204.
22. Futaki S, Kitagawa K. *Tetrahedron Lett.* 1994; 35:1267–1270.
23. Futaki S, Kitagawa K. *Tetrahedron.* 1997; 53:7479–7492.
24. Fontana A, Marchior F, Moroder L, Scoffone E. *Tetrahedron Lett.* 1966:2985–2987.
25. Scoffone E, Fontana A, Rocchi R. *Biochemistry.* 1968; 7:971–979. [PubMed: 4297965]

Figure 1.

Characterization of polypeptides **7** and **9-11**. Panels A-D illustrate RP-HPLC analyses following injection of 1 nmol of peptide eluted with 0.1 v/v % TFA and an acetonitrile gradient of 5-55 % over 30 min while monitoring Abs_{280 nm}. The insets show the ESI-MS for the product peaks. Panel A-C illustrate the re-analysis of preparatively purified **7**, **9**, and **10**, each containing 4 peptide subunits, following convergent reducible ligation. Panel D illustrates the re-analysis of preparatively purified **11** ligated through 7 disulfide bonds. Peaks a-f mark the $[M+7H]^+$ - $[M+12H]^+$ charge states of 1825.1, 1597.4, 1419.6, 1278.0, 1161.5, and 1065.1 m/z respectively.

Scheme 1.

Synthesis of polypeptide **7**: (a) 100 mM ammonium acetate, pH 5; (b) 100 mol eq AgOTf in 96:4 TFA:anisole; (c) i. 5 v/v % piperidine/DMF; ii. 50 mol eq AgOTf in 0.1 v/v % TFA; iii. 10 mol eq DTDP in 10:3 2 M acetic acid:isopropanol; (d) 100 mM ammonium acetate, pH 5 with 0.5 w/v % ammonium chloride.

Scheme 2.

Synthesis of polypeptide **11**: (a) 100 mM ammonium acetate, pH 5; (b) i. 5 v/v % piperidine/DMF; ii. 50 mol eq AgOTf in 0.1 v/v % TFA; iii. 10 mol eq DTDP in 10:3 2 M acetic acid:isopropanol; iv. 0.67 mol eq **6a** in 100 mM ammonium acetate, pH 5 with 0.5 w/v % ammonium chloride; (c) 100 mol eq AgOTf in 96:4 TFA:anisole; (d) i. 100 mol eq AgOTf in 96:4 TFA:anisole; ii. 1.5 mol eq **5b** in 100 mM ammonium acetate, pH 5 with 0.5 w/v % ammonium chloride; (e) (i) 5 v/v % piperidine/DMF; ii. 50 mol eq AgOTf in 0.1 v/v % TFA; iii. 10 mol eq DTDP in 10:3 2 M acetic acid:isopropanol; (f) 100 mM ammonium acetate, pH 5 with 0.5 w/v % ammonium chloride

Table 1

Synthetic yields and mass spectral characterization of peptides and polypeptides

Peptide ^a	Mass (calc / obs) ^b	% Yield
H-Cys-Trp-(Lys) ₁₀ -OH (1)	1589.1 / 1589.2	28 ^c
Fmoc-Thz-Trp-(Lys) ₉ -Cys(Pys)-OH (2)	1907.5 / 1907.2	33 ^c
H-Cys-Trp-(Lys) ₉ -Cys(Acm)-OH (3)	1635.1 / 1635.2	32 ^c
Fmoc-Trp-(Lys) ₁₀ -Cys(Pys)-OH (4)	1920.5 / 1920.8	36 ^c
Fmoc-Thz-Trp-(Lys) ₉ -Cys-OH/H-Cys-Trp-(Lys) ₁₀ -OH (5)	3385.4 / 3384.9	71 ^d
Fmoc-Trp-(Lys) ₁₀ -Cys-OH/H-Cys-Trp-(Lys) ₉ -Cys(Acm)-OH (6)	3444.5 / 3444.0	49 ^d
Fmoc-Trp-(Lys) ₁₀ -Cys-OH/(H-Cys-Trp-(Lys) ₉ -Cys-OH) ₂ /H-Cys-Trp-(Lys) ₁₀ -OH (7)	6522.5 / 6520.9	74 ^d
Fmoc-Thz-Trp-(Lys) ₉ -Cys-OH/H-Cys-Trp-(Lys) ₉ -Cys(Acm)-OH (8)	3431.4 / 3431.4	81 ^d
Fmoc-Thz-Trp-(Lys) ₉ -Cys-OH/(H-Cys-Trp-(Lys) ₉ -Cys-OH) ₂ /H-Cys-Trp-(Lys) ₁₀ -OH (9)	6509.5 / 6508.5	32 ^d
Fmoc-Trp-(Lys) ₁₀ -Cys-OH/(H-Cys-Trp-(Lys) ₉ -Cys-OH) ₂ /H-Cys-Trp-(Lys) ₉ -Cys(Acm)-OH (10)	6568.5 / 6567.1	54 ^d
Fmoc-Trp-(Lys) ₁₀ -Cys-OH/(H-Cys-Trp-(Lys) ₉ -Cys-OH) ₆ /H-Cys-Trp-(Lys) ₁₀ -OH (11)	12770.7 / 12768.7	17 ^d

^aThz refers to thiazolidine, Acm to acetamidomethyl, and Pys to 2-sulfanylpuridine.^bThe observed mass was calculated as the average from the deconvoluted masses of the observed multiple charged ions.^cPurified yield based on initial resin substitution.^dPurified yield determined for the last ligation step.