Cyclic Peptides

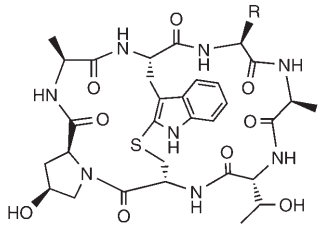
DOI: 10.1002/anie.200700017

A Practical Solid-Phase Synthesis of Glu^7-Phalloidin and Entry into Fluorescent F-Actin-Binding Reagents**

*Laura A. Schuresko and R. Scott Lokey**

Phalloidin (**1**) is a potent actin-binding toxin whose chemistry and bioactivity have been studied since the early 1900s.[1] Phalloidin binds with high affinity to filamentous actin (F-actin) and lowers the critical concentration of actin polymerization in solution.[2] It has been used extensively to study actin dynamics in vitro, and fluorescent analogues of phalloidin serve as highly specific reagents for microscopic visualization of the actin cytoskeleton.[3] The natural source of phalloidin, *Amanita phalloides*, is a mushroom that lives in a complex ecological relationship with associated flora and is widely considered to be uncultivable.[1] Thus, an efficient synthetic route to phalloidin would be highly desirable as an alternative source for this reagent and as an entry into related cyclic peptides. Although there have been a number of syntheses of phalloidin analogues both in solution[4–7] and on a solid phase,[8] no synthetic route has been published with yields significant enough to provide this reagent in practical quantities. The yields reported for these syntheses, which relied on the preparation of relatively complex building blocks in solution, ranged from 0.5[4] to 1.3 %.[6,8] Herein we report an efficient and practical solid-phase synthesis of Glu^7-phalloidin (**2**) in 50 % overall yield from simple starting materials. Derivatization of the Glu^7 side chain yielded a fluorescent analogue that stains F-actin in fixed cells at a concentration comparable to that of commercial phalloidin-based probes.

Phalloidin is a bicyclic heptapeptide which contains an unusual bridging thioether linkage between the Cys and Trp residues. The natural product contains four common L-amino acid residues, a D-threonine residue, an unusual γ,δ-dihydroxy-L-leucine residue, and the rare *cis* epimer of 4-hydroxy-L-proline. Structure–activity studies have shown that the γ,δ-dihydroxy-L-leucine side chain is not essential for actin binding.[7,9] We therefore replaced this residue with glutamic acid to provide the derivative **2** with both a handle for linkage to the solid phase and a site for fluorophore attachment. The *cis*-4-hydroxy-L-proline residue was prepared according to published methods.[8,10]



phalloidin (**1**) Glu^7-phalloidin (**2**)

Central to any total synthesis of phalloidin and its derivatives is the formation of the transannular thioether bridge between the Cys and Trp residues. This transformation has been effected in previous syntheses by the treatment of a cysteine-derived sulfenyl chloride with a suitably protected tryptophan derivative,[8,11,12] or through the attack of a cysteine sulfhydryl group on an oxidatively activated tryptophan indole.[13,14] Although these syntheses provided efficient access to the Trp–Cys thioether adduct in solution, there has been no report of the direct thionation of a Trp indole on the solid phase.

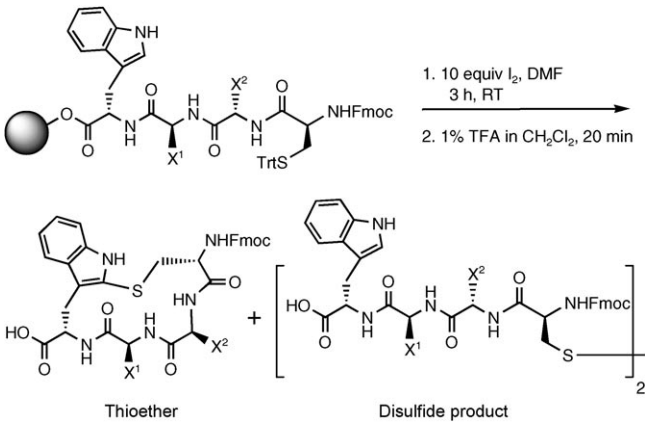
Our approach to the formation of the critical thioether bridge was inspired by a side reaction reported to occur during solution-phase I_2-mediated deprotection of *S*-tritylcysteine (Cys(Trt)) in peptides containing tryptophan.[15] The minor product resulted from thioether formation between the Cys and Trp residues, which occurred presumably through the attack of the tryptophan indole on the sulfenyl iodide intermediate that forms upon trityl deprotection.[16] By using peptides based on the sequence Cys(Trt)-Gly$_n$-Trp, the same authors showed that I_2 treatment in dilute solution led to thioether formation that was more efficient than disulfide dimerization for $n > 3$.

When we applied similar conditions to the solid-phase synthesis of model peptides based on the thioether-containing sequence of phalloidin, the only observed products were the desired thioether and the dimer resulting from on-resin intermolecular disulfide formation. For the sequence H_2N-Cys-Pro-Ala-Trp-OH, at a loading value of 0.1 mmol g^{-1}, cyclization was twice as efficient as dimerization (Scheme 1). When the L-proline residue was replaced with TIPS-protected *cis*-4-hydroxy-L-proline, the ratio of the thioether to the disulfide product increased to 6.6:1. These results encouraged us to undertake a solid-phase synthesis of Glu^7-phalloidin with an I_2-mediated cyclization as the key step to form the thioether bridge.

[*] L. A. Schuresko, Dr. R. S. Lokey
Department of Chemistry and Biochemistry
University of California Santa Cruz
Santa Cruz, CA, 95064 (USA)
Fax: 831-459-2935
E-mail: lokey@chemistry.ucsc.edu

[**] The support of the NIH (1RO1 CA104569-01) for this research is gratefully acknowledged. We also thank Craig Tamble for assistance in cell culture and actin staining.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



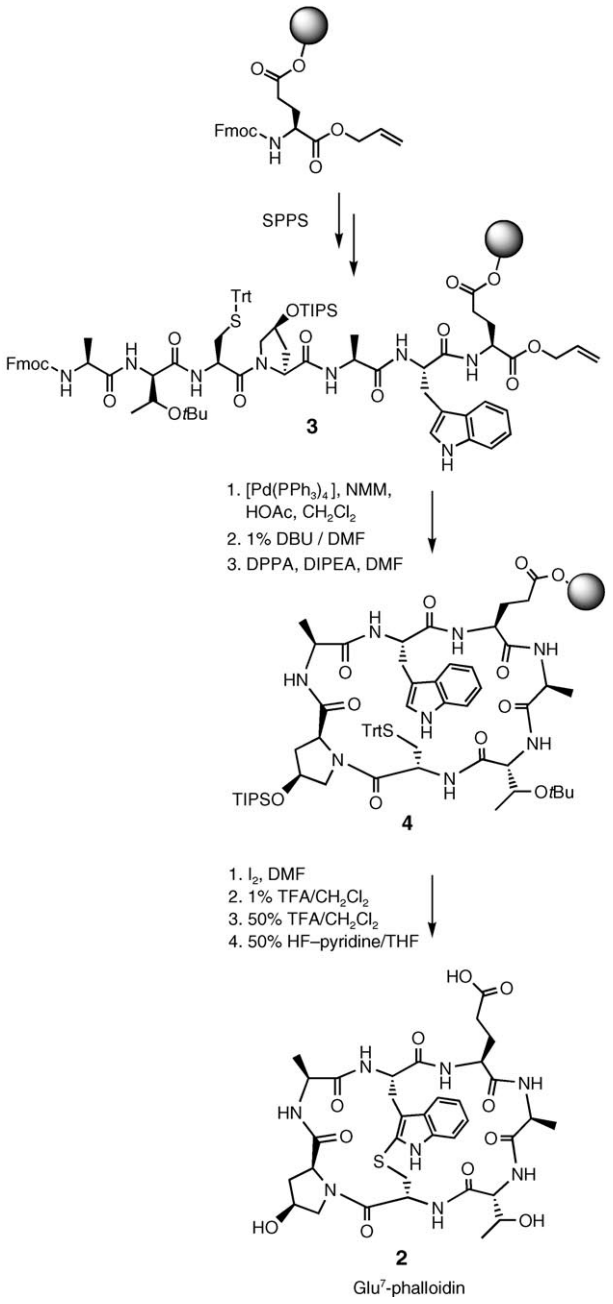
Scheme 1. Assessment of solid-phase I_2-mediated cyclization as a strategy for the construction of the thioether bridge in phalloidin derivatives by using tetrapeptide model systems. The thioether/disulfide product ratios are 2:1 (for Fmoc-NH-Cys-Pro-Ala-Trp-OH) and 6.6:1 (for Fmoc-NH-Cys-Hyp(OTIPS)-Ala-Trp-OH). DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, TFA = trifluoroacetic acid, TIPS = triisopropylsilyl.

To generate the appropriate peptide precursor, the Glu^7 residue was protected at its C terminus as an allyl ester and linked through its side chain to 2-chlorotrityl polystyrene resin. Elongation to give the heptapeptide was carried out by using standard Fmoc chemistry (Scheme 2), and after removal of the N-terminal Fmoc group[17] and C-terminal allyl ester, the peptide backbone was cyclized by treatment with diphenylphosphorylazide (DPPA). Following cleavage of the product from the resin, HPLC–MS analysis showed that the macrolactamization had proceeded efficiently. No cyclodimer or higher oligomers were observed.

When the resin-bound cyclic peptide **4** was treated with I_2 in DMF, complete conversion into the thioether was observed, with no intermolecular disulfide dimer detected. Upon cleavage from the resin with 1% trifluoroacetic acid/CH_2Cl_2, the crude, fully protected cyclic product was found to be more than 90% pure by LC–MS; no higher oligomers were present. Removal of the protecting groups on the D-Thr and 4-hydroxy-L-proline side chains by sequential treatment with TFA/CH_2Cl_2 (1:1) and 50% HF–pyridine in THF, and purification by reversed-phase HPLC, yielded a single major product whose circular dichroism (CD) and ^{1}H NMR spectra[8] were consistent with Glu^7-phalloidin (see the Supporting Information for complete characterization). The overall yield of the purified material was 50% on the basis of the initial resin loading.

Synthetic phallotoxins can exist as two isolatable atropisomers.[1] The synthetic route reported herein provides exclusive access to the natural atropisomer, as determined by comparison of the CD spectrum of Glu^7-phalloidin with that of the authentic natural product. Furthermore, the distinctive upfield chemical shift of the Ala^5 methyl group is diagnostic of the natural atropisomer because of the proximity of this group to the anisotropy field of the tryptophan indole ring.[8]

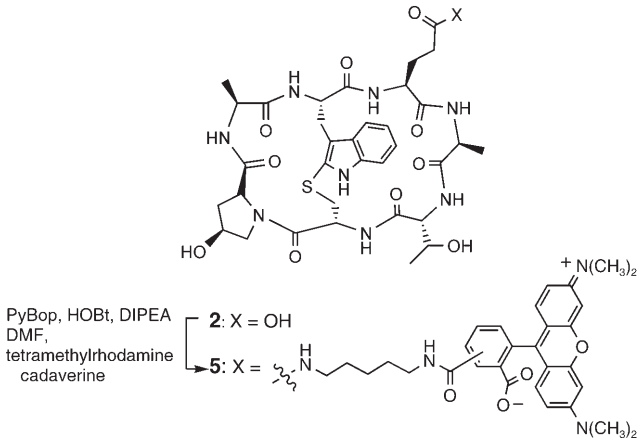
Tetramethylrhodamine cadaverine was conjugated to compound **2** by using PyBOP/HOBt in DMF to yield the



Scheme 2. Solid-phase peptide synthesis (SPPS) of Glu^7-phalloidin. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DIPEA = *N*,*N*′-diisopropylethylamine, NMM = *N*-methylmaleimide.

fluorescent adduct **5** (Scheme 3). Cultured mammalian epithelial (BS-C-1) cells were fixed and treated with **5** at 1 $\mu g\,mL^{-1}$ by following standard protocols (see the Supporting Information). Fluorescence microscopy revealed the F-actin staining pattern typical of commercial phalloidin conjugates (Figure 1). Actin-filament staining was completely abolished when the fixed cells were pretreated with natural phalloidin, thus demonstrating the specificity of **5** for F-actin.

In summary, we have developed a simple synthesis of Glu^7-phalloidin and its derivatives, including a fluorescent bioactive probe that is as effective as natural phalloidin conjugates in staining F-actin in fixed cells. This route will



Scheme 3. Conversion of Glu[7]-phalloidin (**2**) into a rhodamine derivative. HOBt = 1-hydroxy-1*H*-benzotriazole, PyBOP = benzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate.

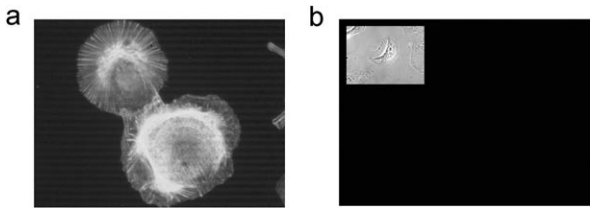


Figure 1. a) Fluorescence micrographs of a) BS-C-1 cells stained with the tetramethylrhodamine–phalloidin derivative **5** and b) cells pretreated with natural phalloidin (**1**) prior to the addition of **5**; inset shows a phase-contrast image of the same field.

generate sufficient amounts of fluorescently labeled phalloidin to perform high-throughput image-based screens for compounds that affect actin morphology, and will allow us to make extensive modifications to the phalloidin scaffold for future structure–activity studies.

Received: January 3, 2007
Published online: March 27, 2007

Keywords: cyclization · fluorescent probes · natural products · peptides · solid-phase synthesis

[1] T. Wieland, *Peptides of Poisonous Amanita Mushrooms*, Springer, New York, **1986**.
[2] J. E. Estes, L. A. Selden, L. C. Gershman, *Biochemistry* **1981**, *20*, 708.
[3] J. R. Pringle, A. E. Adams, D. G. Drubin, B. K. Haarer, *Methods Enzymol.* **1991**, *194*, 729.
[4] E. Munekata, H. Faulstich, T. Wieland, *Justus Liebigs Ann. Chem.* **1977**, 1758.
[5] E. Wulf, A. Deboben, F. A. Bautz, H. Faulstich, T. Wieland, *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 4498.
[6] G. Zanotti, L. Falcigno, M. Saviano, G. D'Auria, B. M. Bruno, T. Campanile, L. Paolillo, *Chem. Eur. J.* **2001**, *7*, 1479.
[7] L. Falcigno, S. Costantini, G. D'Auria, B. M. Bruno, S. Zobeley, G. Zanotti, L. Paolillo, *Chem. Eur. J.* **2001**, *7*, 4665.
[8] M. O. Anderson, R. K. Guy, *J. Org. Chem.* **2005**, *70*, 4578.
[9] T. Wieland, *Int. J. Pept. Protein Res.* **1983**, *22*, 257.
[10] C. A. Weir, C. M. Taylor, *J. Org. Chem.* **1999**, *64*, 1554.
[11] T. Wieland, C. Jochum, H. Faulstich, *Justus Liebigs Ann. Chem.* **1969**, *727*, 138.
[12] T. Wieland, O. Weiberg, E. Fischer, G. Horlein, *Liebigs Ann. Chem.* **1954**, *587*, 146.
[13] W. E. Saviage, A. Fontana, *Int. J. Pept. Protein Res.* **1980**, *15*, 102.
[14] W. E. Saviage, A. Fontana, *J. Chem. Soc. Chem. Commun.* **1976**, 600.
[15] P. Sieber, B. Kamber, B. Riniker, W. Rittel, *Helv. Chim. Acta* **1980**, *63*, 2358.
[16] Alternatively, thionation may occur by iodination of the indole to form a 3-iodoindolenine intermediate, which undergoes nucleophilic attack at C2 by the sulfur atom followed by dehydrohalogenation.
[17] Initial attempts to remove the final Fmoc group by using 20% piperidine in DMF resulted in the formation of unidentified side products and a low overall yield of Glu[7]-phalloidin. Treatment of the linear peptide with 1% DBU in DMF, however, led to clean deprotection of the N terminus and a dramatic increase in the yield of the final product.

