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# A Solid-Phase Approach to the Phallotoxins: Total Synthesis of [Ala$^7$]-Phalloidin

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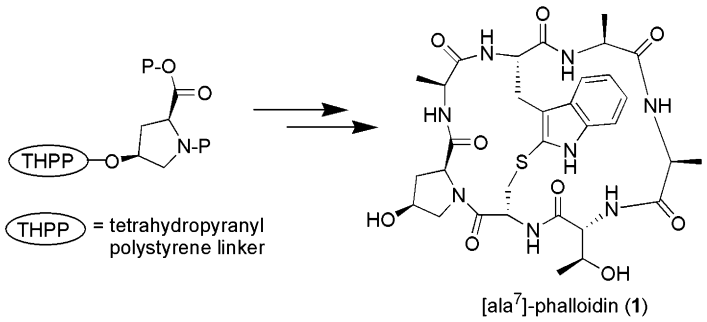


[ala$^7$]-phalloidin (**1**)

Herein we report a solid-phase synthetic approach to [Ala$^7$]-phalloidin (**1**). Prior syntheses of phallotoxins were carried out using solution-phase routes that required large scale and preclude library production. The route presented here consists of solution-phase preparation of key orthogonally protected amino acid building blocks, followed by a solid-phase peptide synthesis sequence, featuring two resin-bound macro-cyclization reactions. The final product mixture was composed of two atropisomeric compounds, one designated "natural" (**1**) and the other designated "non-natural" (**1′**). The structures of these species were modeled using restrained energy minimization with NMR-derived restraints.

## Introduction

Phalloidin (**2**) is a cyclic heptapeptide fungal toxin produced by *Amanita phalloides* (the Death Cap mushroom).[1] This compound is a potent stabilizer of filamentous actin, and fluorescently labeled phallotoxins have been used in cellular biology to study the biochemistry of the actin system.[1a,2] The actin polymerization equilibrium plays an important role in cellular processes such as signaling,[3] motility,[4] endocytosis,[5] tumorigenesis,[6] and metastasis.[7] Actin has been suggested as a target for cancer chemotherapy.[7b]

Although the biochemistry and toxicology of phalloidin (**2**) have been explored for nearly 200 years, a total synthesis of this compound remains to be reported. A notable amount of synthetic work on phalloidin as well as natural and non-natural analogues of this compound (phallotoxins) was carried out by Wieland et al.[1a,8] More recently, Paolillo et al. published a synthesis of [Ala$^7$]-phalloidin (**1**)[9] and several other analogues.[10]

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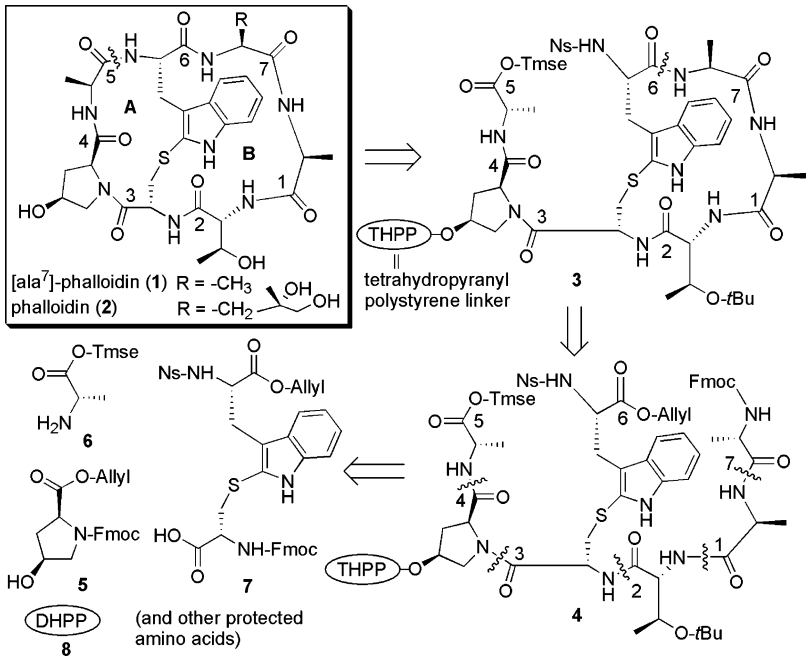


**FIGURE 1.** Retrosynthetic analysis of [Ala⁷]-phalloidin (**1**). The route is designed to allow for solid-supported synthesis of libraries with maximal diversity.

The previous synthetic routes to the phallotoxins relied upon large-scale solution-phase peptide synthesis techniques. Our own retrosynthetic analysis suggested that a solid-phase approach might be advantageous for the rapid and efficient construction of the phallotoxins. In particular, such an approach would allow access to a diverse library of compounds. Herein we report a novel solid-phase approach to the phallotoxins, namely, the total synthesis of [Ala⁷]-phalloidin (**1**).

## Results and Discussion

A retrosynthesis of [Ala⁷]-phalloidin (**1**) is presented in Figure 1. The molecule is a bicyclic heptapeptide with an unusual Trp⁶-Cys³ thioether unit bridging the molecule. Two interesting features are the Hyp⁴ residue, which has the rare *cis* relative configuration, and Thr², which has the less common D-stereochemistry at the α-carbon. We envisaged the synthesis of [Ala⁷]-phalloidin (**1**) proceeding with the compound anchored to a solid support through the *cis*-Hyp⁴ side chain using the acid-labile tetrahydropyranyl polystyrene linker (THPP).[11] The A ring was opened early in the retrosynthesis, between residues Trp⁶ and Ala⁵, leading to resin-bound intermediate **3**. In intermediate **3**, the Ala⁵ carboxy group was protected as a fluoride-labile 2-trimethylsilylethyl (Tmse) ester[12] and the Trp⁶ amino group was protected as the thiolate-labile *o*-nitrobenzenesulfonamide (Ns).[13] The Thr² side chain was protected as an acid-labile *tert*-butyl ether group, allowing simultaneous resin cleavage and side chain deprotection in the forward direction. Next in the retrosynthesis, the B ring was opened between residues Ala⁷ and Trp⁶, leading to precursor **4**. Strategically, our decision for the order of cyclizing the A and B rings, as well as the specific residues to cyclize, was based on precedent in the solution-phase phallotoxin synthesis literature.[8c,9,10] Disconnection of precursor **4** using standard peptide synthesis transformations gives rise to species **5**−**7**, the free solid support **8**, and other commercially available protected amino acids.

Compound **5** was prepared through Mitsunobu reaction of a corresponding inexpensive *trans*-hydroxyproline derivative, using conditions previously developed for the inversion of this class of compounds[14] (see Figure S1, Supporting Information). Compound **6** was available in two steps from commercially available Cbz-Ala-OH.[15]

The synthesis of the central Trp⁶-Cys³ unit (**7**) is depicted in Figure 2. Commercially available protected cystine **9** was transformed to di-*tert*-butoxycarbamate **10**.[16] Next, disulfide **10** was oxidatively cleaved with sulfuryl chloride[17] to generate 2 equiv of the corresponding sulfenyl chloride **11**. This species was then im-

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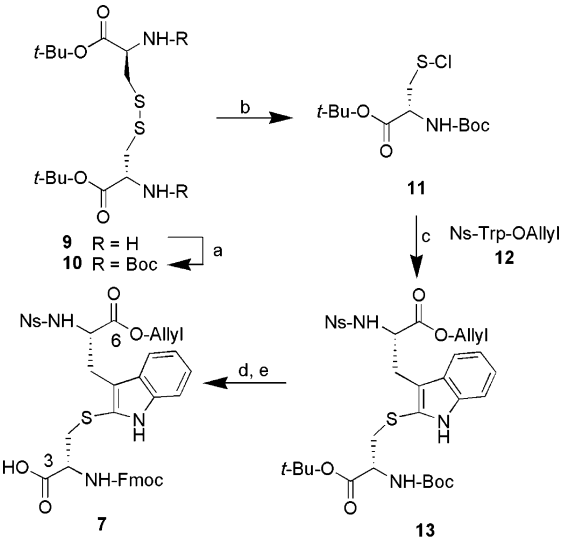

**FIGURE 2.** Synthesis of intermediate **7**. Reagents and conditions: (a) di-*tert*-butyl dicarbonate, MeOH, $Et_3N$, 10 min, 60 °C, 95%; (b) $SO_2Cl_2$, $CHCl_3$, under air, 45 min, room temperature; (c) (*o*-$NO_2$Ph)$SO_2$-Trp-*O*-Allyl (**12**), $NaHCO_3$, $CHCl_3$, under argon, 15 min, room temperature (71% two steps); (d) $CF_3CO_2H$, (*i*-Pr)$_3$SiH, 24 h, room temperature; (e) 9-fluorenylmethyl chloroformate, $Na_2CO_3$ (1 M aq), dioxane, 2 h, 0 °C (66% two steps).

mediately coupled[8b,18] with orthogonally protected tryptophan fragment **12** to yield thioether **13**. In our exploration of this two-step process, it was found that the generation of sulfenyl chloride **11** must be carried out under an atmosphere of air and that the reaction does not proceed under inert atmosphere (e.g., argon). We speculate that the transformation of a disulfide to a sulfenyl chloride using sulfuryl chloride may occur by a radical mechanism requiring an initiator (in this case, oxygen diradical). Finally, removal of the acid-labile Cys[3] protecting groups, followed by reprotection of the amino group, was carried out to arrive at target fragment **7**.

The initial steps of the solid-phase synthesis sequence are illustrated in Figure 3. First, dihydropyranyl polystyrene resin **8** was loaded with protected *cis*-Hyp[4] **5** to yield tetrahydropyranyl polystyrene-bound intermediate **14** (53% loading by mass of the resulting resin). $Pd^0$-catalyzed deprotection of the allyl ester gave the free acid. Coupling with protected Ala[5] **6** in the presence of (benzotriazole-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP),[19] 1-hydroxybenzotriazole (HOBt), and (*i*-Pr)$_2$EtN, generated dipeptide **15**. The Hyp[4] Fmoc group was removed to generate the free secondary amine. To limit the recognized problem of racemization when coupling to carbamate-protected cysteines,[20] the reagents (7-azabenzotriazole-1-yloxy) tripyrrolidino-phosphonium hexafluorophosphate (PyAOP), 1-hydroxy-7-azabenzotriazole (HOAt), and the weak base 2,4,6-collidine were then used to effect coupling of **15** with acid **7** to generate

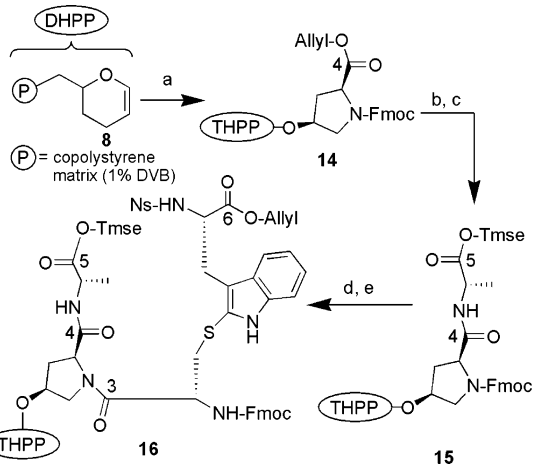

**FIGURE 3.** Synthesis of tetrapeptide **16**. Reagents and conditions: (a) Fmoc-*cis*-Hyp-*O*-Allyl (**5**), pyridinium *p*-toluenesulfonate, 1,2-dichloroethane, 18 h, 80 °C; (b) $Pd(PPh_3)_4$, *N*,*N*-dimethylbarbituric acid, $CH_2Cl_2$, 2 h, room temperature; (c) *H*-Ala-*O*-Tmse (**6**), (benzotriazole-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxy-benzotriazole (HOBt), (*i*-Pr)$_2$EtN, DMF/$CH_2Cl_2$ (1:1), 2 × 30 min, room temperature; (d) piperidine (20% in DMF), 10 min, room temperature; (e) Fmoc-Cys-[*S*-(2-((*o*-$NO_2$Ph)$SO_2$-Trp-*O*-Allyl))]-OH (**7**), (7-azabenzotriazole-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP), 1-hydroxy-7-azabenzotriazole (HOAt), 2,4,6-collidine, DMF/$CH_2Cl_2$ (1:1), 30 min, room temperature.

tetrapeptide **16**. Progress of the reactions was generally confirmed by qualitative bead-analysis tests, such as the standard Kaiser and chloranil tests, as well as microscale cleavage followed by RP-HPLC and either MALDI-TOF or ESI-MS analysis.

The next sequence of steps is shown in Figure 4. Iterative Fmoc peptide synthesis was carried out on intermediate **16** to attach the D-Thr[2], Ala[1], and Ala[7] residues, yielding compound **4**. The allyl ester and Fmoc groups were removed by $Pd^0$-catalyzed trans-allylation and by treatment with base, respectively. Two sets of conditions were examined for the subsequent cyclization: (1) PyAOP,[21] HOAt, (*i*-Pr)$_2$EtN, DMF/$CH_2Cl_2$, 24 h; and (2) diphenylphosphoryl azide,[22] (*i*-Pr)$_2$EtN, DMF, 24 h. After microscale cleavage and analysis by RP-HPLC and MALDI-TOF-MS, both sets of conditions showed a single homogeneous species without evidence of epimerization or cyclodimerization. The less toxic reagent PyAOP was chosen to effect the cyclization and generate **3**.

The final steps to be carried out were deprotection of the Ala[5]-Tmse and Trp[6]-Ns groups, cyclization of the A ring, resin-cleavage, and deprotection to generate the product **1**. Removal of the Ala[5]-Tmse group was affected smoothly using tetrabutylammonium fluoride (TBAF). Deprotection of the Trp[6]-Ns group was surprisingly difficult. Typical reported solid-phase conditions are

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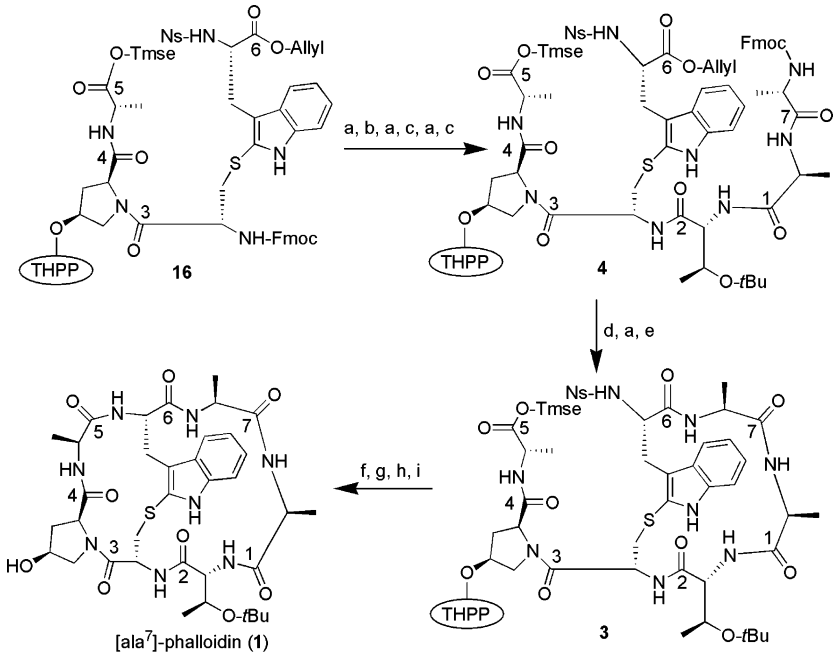


**FIGURE 4.** Synthesis of [Ala⁷]-phalloidin (**1**). Reagents and conditions: (a) piperidine (20% in DMF), 20 min, room temperature; (b) Fmoc-D-Thr(O-*t*-Bu)-OH, PyBOP, HOBT, $(i\text{-Pr})_2$EtN, DMF/$CH_2Cl_2$ (1:1), 30 min, room temperature; (c) Fmoc-Ala-OH, PyBOP, HOBT, $(i\text{-Pr})_2$EtN, DMF/$CH_2Cl_2$ (1:1), 30 min, room temperature; (d) $Pd(PPh_3)_4$, *N*,*N*-dimethylbarbituric acid, $CH_2Cl_2$, 2 h, room temperature; (e) PyAOP, HOAt, $(i\text{-Pr})_2$EtN, DMF/$CH_2Cl_2$ (1:1), 24 h, room temperature; (f) tetrabutylammonium fluoride (1 M in THF) 2 × 24 h, room temperature; (g) $\beta$-mercaptoethanol, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), DMF under argon, 6 × 30 min, room temperature; (h) diphenylphosphoryl azide, $(i\text{-Pr})_2$EtN, DMF, 48 h, room temperature. (i) $CF_3CO_2H/H_2O/Et_3SiH$ (8:2:10), 30 min, room temperature.

treatment with $\beta$-mercaptoethanol and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) under argon.[13d,e] Achieving complete deprotection using these conditions required six successive iterations, presumably because of difficulty in removing residual oxygen from the resin, thus resulting in the oxidation of the thiol reagent.

Conditions were then examined for the cyclization of the A ring. The closure of this ring was more difficult than for the B ring. Three sets of conditions were examined: (1) PyAOP,[21] HOAt, $(i\text{-Pr})_2$EtN, DMF/$CH_2Cl_2$, 48 h; (2) (benzotriazol-1-yloxy)-dipyrrolidinocarbenium hexafluoro-phosphate (HbPyU),[8,9,23] $(i\text{-Pr})_2$EtN, DMF: $CH_2Cl_2$, 48 h; and (3) diphenylphosphoryl azide,[22] $(i\text{-Pr})_2$EtN, DMF, 48 h. After cleavage and analysis by RP-HPLC, it was found that PyAOP and HbPyU were both unsuccessful, showing neither consumption of starting material nor formation of product. Interestingly, HbPyU has been used successfully for solution-phase cyclization of phallotoxins to form the same amide bond.[9,10] Fortunately, diphenylphosphoryl azide effected complete conversion of starting material to cyclized product, although a degree of side-product formation was observed, including suspected oligomers.

Finally, concomitant cleavage from the resin and deprotection of the D-Thr² side chain *tert*-butyl ether group was accomplished with $CF_3CO_2H/H_2O/Et_3SiH$ (8:2:10). Careful analysis of the crude material by RP-HPLC showed that the product was composed of two species with very similar retention times, in a ~1:2 ratio.

RP-HPLC afforded the two species **1** and **1′**. Both compounds were shown by MALDI-TOF-MS to have the same molecular weight as the desired product and seemed likely to be atropisomers of [Ala⁷]-phalloidin, generated in the final cyclization. Species **1** and **1′** were characterized by $^1$H NMR, HMQC, DQF-COSY, and ROESY. All $^1$H spin systems were assigned by comparison of DQF-COSY and ROESY spectra (Tables S1 and S3), and all protonated carbons were assigned by correlation with the HMQC spectra (Tables S2 and S4).

Species **1** possessed spectroscopic data consistent with [Ala⁷]-phalloidin as previously characterized,[9] whereas **1′** had noticeably different data. On the basis of these observations, species **1** was tentatively designated the "natural" atropisomer of [Ala⁷]-phalloidin, whereas species **1′** was designated the "non-natural" atropisomer.

Atropisomeric products have been reported before in cyclizations to form phallotoxins. For example, in the solution-phase synthesis of [L-Thr², Ala⁷]-phalloidin,[10] two species were isolated and identified as atropisomers by spectroscopic techniques and computational modeling. By circular dichroism (CD) analysis, one species was found to be consistent with the natural bioactive phallotoxins,[1a] whereas the other had a spectrum that was generally inverted. Thus, CD analysis was performed on isolated species **1** and **1′**, as well as on authentic phalloidin (**2**) (Figure 5). Phalloidin (**2**) and species **1** both showed positive Cotton effects at 250 and 305 nm. This contrasts with the CD spectrum of species **1′**, which showed negative Cotton effects at the same wavelengths. That the CD spectra of **1** and **1′** are not perfectly symmetric is expected, as the species are not enantiomeric. The similarity in CD of **1** with phalloidin (**2**) suggests that this species possesses the positive helicity of the natural bioactive phallotoxins, whereas **1′** possesses non-natural

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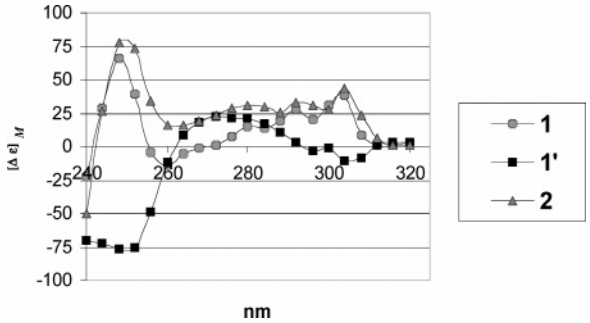


**FIGURE 5.** CD spectra of [Ala[7]]-phalloidin atropisomers (**1** and **1′**) and authentic phalloidin (**2**) (in $CH_3OH$ at 20 °C). The spectra were recorded at 0.2, 0.6, and 0.6 mmolar, respectively, and normalized to determine molar elipticity $[\Delta \epsilon]_M$.

negative helicity. It should be noted that biosynthesized phallotoxins are produced exclusively in positive helical form and that the negative helicity of the latter species is not observed in nature.

The purified yields of the two atropisomeric species **1** and **1′** were 1.3% and 3.2%, respectively, based on 53% initial loading of hydroxyproline **5** onto DHPP resin. While low, these yields can partially be accounted for by the difficulty that was faced in separating the two isomeric products of very similar polarity. The purity of the intermediates throughout the synthesis did seem quite high, implying that most couplings and deprotections proceeded with good conversion (see RP-HPLC traces in Supporting Information). We suggest that the two sequential cyclization reactions (uncommon in peptide synthesis), and particularly the more problematic second cyclization (Figure S20 in Supporting Information) also help to account for the low yield. Furthermore, it should be considered that for a solid-phase synthesis consisting of 18 steps, an overall yield of 1.3% corresponds to an average of 79% yield per step, which is not unusual in natural product synthesis. Last, it is notable that the overall yield we report is comparable to the previous solution-phase synthesis of [Ala[7]]-phalloidin[9] (eight linear steps, 1.3% overall).

Restrained energy minimizations were performed to gain insight into the structures of **1** and **1′**. NH−$H_\alpha$ torsional angle and qualitative distance restraints were derived from DQF-COSY *J*-value and ROESY NMR data, respectively. To model species **1**, the computational starting point was the solution structure of [Ala[7]]-phalloidin established by Paolillo et al.[9] (method I). This structure was minimized over 10 000 steps using our own NMR-derived restraints, yielding a model consistent with the previously reported structure[9] (Figure 6, Tables S5 and S6). By convention, the atropisomerism of bicyclic peptides is designated "U" or "D" on the basis of the orientation of the bridge (up or down) when the peptide is observed in a clockwise manner. The model had a U-type conformation with a positive indole-thioether angle, consistent with the bioactive phallotoxins.[1a] In addition, the anisotropy field of the Trp[6] indole ring is located in the vicinity of the Ala[5] methyl, which accounts for the upfield shift of this group (Table S1). This phenomenon is generally associated with the bioactive phallotoxins.[1a]

When method I was applied to model **1′**, the resulting structure had a U-type conformation and a positive indole-thioether angle. However, the validity of this model was questioned because of the relatively high total energy and the significant violation of torsional angle and distance restraints (Table S7). Furthermore, the "inverted" CD spectrum observed for **1′** is inconsistent with a positive indole-thioether angle, based on X-ray analysis of early crystalline phalloidin analogues.[1a,24]

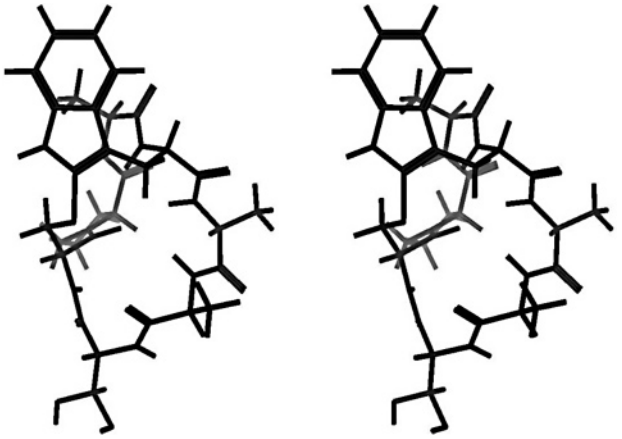

**FIGURE 6.** Stereo drawing of the [Ala[7]]-phalloidin "natural" atropisomer **1** derived from computational method I.

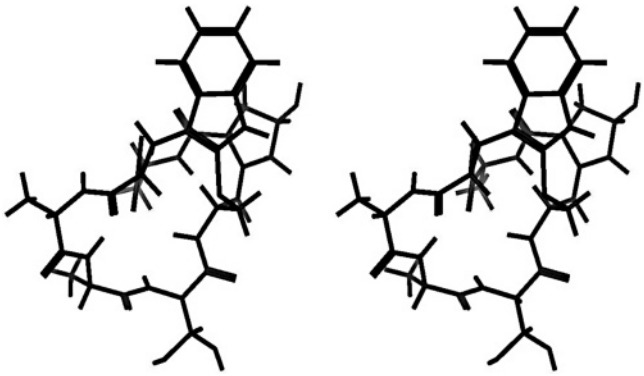

**FIGURE 7.** Stereo drawing of the [Ala[7]]-phalloidin "non-natural" atropisomer **1′** derived from computational method II.

The first method was then modified to assume an initial inverted D-type structure (method II). This simulation produced a reasonable model of species **1′** (Figure 7 and Tables S8 and S9) with significantly less violation and lower total energy than the previous model. Notably, the model had a negative indole-thioether angle, as expected on the basis of the inverted CD spectra of this species.[1a,24] Furthermore, this model places the anisotropy field of the Trp[6] indole residue near Hyp[4]-$H_\alpha$ and Cys[3]-$H_\alpha$, accounting for the relative upfield shifts of these protons (Table S3). Overall, this model of **1′** is fairly consistent with a model proposed for a "non-natural" atropisomer of [L-Thr[2], Ala[7]]-phalloidin,[10] a species also showing upfield shifts of Hyp[4]-$H_\alpha$ and Cys[3]-$H_\alpha$. An interesting mechanistic possibility that may have led to the inverted structure of **1′** is that, during activation preceding the final cyclization, Ala[5] may have epimerized

(24) (a) Wieland, T.; Beijer, B.; Seeliger, A.; Dabrowski, K.; Zanotti, G.; Tonelli, A. E.; Gieren, A.; Dederer, B.; Lamm, V.; Hadicke, E. *Liebigs Ann. Chem.* **1981**, 2318−2334. (b) Zanotti, G.; Beijer, B.; Wieland, T. *Int. J. Pept. Protein Res.* **1987**, *30*, 323−329.

to D-Ala[5], and that cyclization of the resulting peptide could have led to **1′**. In this case, **1′** would be formally described as a conformationally inverted diastereomer of **1**. Indeed, the use of DPPA has been shown to cause racemization in slower cyclizations.[25] As the NMR-RMD approach utilized in these studies cannot conclusively establish stereochemistry, this explanation also seems reasonable and is worth consideration.

## Conclusion

A convergent solid-phase synthesis of [Ala⁷]-phalloidin (**1**) has been reported. These studies demonstrate the rapid solid-phase construction of an orthogonally protected linear peptide sequence followed by two sequential on-resin macrocyclization reactions. The final cyclization was shown to generate two species (**1** and **1′**) believed to be atropisomers. The "natural" atropisomer (**1**) was minimized to a reasonable structure consistent with the previously reported structure of [Ala⁷]-phalloidin. This contrasts with the "non-natural" atropisomer (**1′**), which required an initial starting structure with an overall inverted shape relative to species **1**. In future studies, we plan to reexamine cyclization of the A ring in order to optimize for formation of the natural atropisomer. The extension of this strategy to target phalloidin (**2**), as well as other phallotoxins, will be reported in due course.

## Experimental Section

For general experimental information, see Supporting Information.

**Boc-Cys-[*S*-(2-((*o*-$NO_2$Ph)$SO_2$-Trp-*O*-Allyl))]-*O*-*t*-Bu Ester (13).** A solution of (Boc-Cys-*O*-*t*-Bu)$_2$ (**10**)[26] (1.017 g, 1.84 mmol) in anhydrous chloroform (18 mL) was vigorously stirred under air and then treated dropwise with sulfuryl chloride (0.44 mL, 5.5 mmol, 3.0 equiv). The solution was stirred for 45 min at room temperature, under air, becoming progressively more yellow over this period of time. TLC indicated that the generation of the sulfenyl chloride was mostly complete (5:2 hexanes/ethyl acetate, UV, $I_2$, product $R_f = 0.4$). The solution was quickly concentrated in vacuo to remove excess sulfuryl chloride, yielding the sulfenyl chloride (**11**) as a viscous yellow oil.

The sulfenyl chloride was immediately dissolved in anhydrous $CHCl_3$ (18 mL) and stirred for 5 min in an ice bath, under argon. The solution was quickly treated with $NaHCO_3$ (0.309 g, 3.68 mmol, 2.0 equiv), followed immediately by a solution of (*o*-$NO_2$Ph)$SO_2$-Trp-*O*-Allyl ester (**12**) (0.869 g, 2.02 mmol, 1.1 equiv) in anhydrous $CHCl_3$ (18 mL). The solution was stirred in an ice bath for 15 min and then warmed to room temperature. TLC (5:2 hexanes/ethyl acetate, UV) showed the product ($R_f = 0.3$), as well as a small amount of Ns-Trp-*O*-Allyl ester ($R_f = 0.1$). The solution was concentrated in vacuo on silica. The product was isolated by dry-loaded silica flash column chromatography (5:2 hexanes/ethyl acetate) as a yellow oil (0.916 g, 71%): $[\alpha]^{25}_D$ 91.4 (*c* $CHCl_3$). IR ($CDCl_3$): 3154, 2983, 1733, 1696 cm$^{-1}$. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 1.40 (s, 9H), 1.46 (s, 9H), 2.68 (t, 1H, $J$ = 14 Hz), 3.12−3.35 (mult, 2H), 4.37−4.53 (mult, 4H), 5.12−5.18 (mult, 2H), 5.33 (d, 1H, $J$ = 8 Hz), 5.66−5.67 (mult, 1H), 6.04 (d, 1H), 6.97 (t, 1H, $J$ = 8 Hz), 7.12 (t, 1H, $J$ = 8 Hz), 7.27 (d, 1H, $J$ = 8 Hz), 7.36 (d, 1H, $J$ = 8 Hz), 7.52−7.54 (mult, 2H), 7.63 (dd, 1H, $J$ = 2, 8 Hz), 7.81 (dd, 1H, $J$ = 2, 8 Hz), 10.2 (bs, 1H). $^{13}$C NMR (100 Hz, $CDCl_3$): $\delta$ 28.1, 28.5, 29.1, 41.3, 54.3, 57.6, 66.4, 81.0, 84.1, 111.6, 114.45, 118.6, 119.1, 120.0, 123.2, 125.6, 126.8, 127.3, 130.3, 131.4, 132.8, 133.3, 134.1, 136.8, 147.3, 157.8, 170.1, 170.9. HRMS calcd for $C_{32}H_{40}N_4O_{10}S_2$ [M] 704.2186, found [M] 704.2183.

**Fmoc-Cys-[*S*-(2-((*o*-$NO_2$Ph)$SO_2$-Trp-*O*-Allyl))]-OH (7).** To a solution of Boc-Cys-[*S*-(2-((*o*-$NO_2$Ph)$SO_2$-Trp-*O*-Allyl))]-*O*-*t*-Bu (**13**) (1.137 g, 1.62 mmol) in a minimal amount of $CH_2Cl_2$ (∼5 mL) was added triisopropylsilane (1 mL, 4.84 mmol, 3 equiv) and $CF_3CO_2H/H_2O$ (8:2 v/v) (21 mL). The heterogeneous solution was vigorously stirred for 24 h at room temperature. Low resolution ESI-MS of the crude reaction mixture showed the presence of the desired deprotected material. The solution was concentrated in vacuo and resuspended in a mixture of 1,4-dioxane (16 mL), $Na_2CO_3$ (1 M aq, 16 mL), and $H_2O$ (10 mL). The solution was then stirred in an ice bath for 5 min, and then a solution of 9-fluorenylmethyl chloroformate (0.554 g, 1.2 equiv) in 1,4-dioxane (2 × 5 mL) was added over 5 min. The solution was stirred vigorously for 1 h at 0 °C, with frequent addition of NaOH (1 M aq) to maintain the pH between 9 and 10 (tested with pH paper). To the solution was added brine (100 mL) followed by HCl (1 M aq) to adjust the pH to 2. The crude product was then extracted with $CH_2Cl_2$ (3 × 50 mL), washed with brine (50 mL), dried over $MgSO_4$, and concentrated in vacuo on silica. The product was isolated by dry-loaded silica flash column chromatography (1:1 hexane/ethyl acetate to 1:2 hexanes/ethyl acetate (1% acetic acid)). The product was typically contaminated with acetic acid, which was removed azeotropically by the addition and evaporation of toluene (10 mL × 3), yielding the purified product as a yellow foam (0.826 g, 66%): $[\alpha]^{25}_D = -90.6$ (*c* $CH_2Cl_2$). IR ($CDCl_3$): 3154, 2984, 2901, 1793 cm$^{-1}$. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 2.88−2.94 (mult, 1H), 3.30−3.38 (mult, 3H), 4.17 (t, 1H, $J$ = 7 Hz), 4.35−4.40 (mult, 3H), 4.53−4.56 (mult, 3H), 4.65 (bs, 1H), 5.09−5.16 (mult, 2H), 5.62−5.70 (mult, 1H), 5.75 (d, 1H, $J$ = 8 Hz), 6.17 (d, 1H, $J$ = 8 Hz), 6.93 (t, 1H, $J$ = 8), 7.08 (t, 1H, $J$ = 8 Hz), 7.18−7.46 (mult, 8H), 7.56−7.61 (mult, 3H), 7.72−7.74 (mult, 3H), 9.43 (bs, 1H). $^{13}$C NMR (100 Hz, $CDCl_3$): $\delta$ 27.1, 39.8, 47.2, 54.1, 57.4, 66.6, 67.9, 111.6, 115.1, 118.7, 119.3, 120.2, 120.2, 123.5, 125.4, 125.5, 125.9, 127.3, 127.4, 128.0, 130.2, 131.2, 132.9, 133.4, 133.9, 136.8, 141.5, 143.8, 147.0, 171.1, 177.2. HRMS calcd for $C_{38}H_{34}N_4O_{10}S_2$ [M + H] 771.1794, found [M + H] 771.1792.

**General Procedures for Solid-Phase Synthesis.** (Based on estimated initial loading of 0.217 mmol).

**General Procedure A: Washing of Solid-Phase Resin.** The reaction vessel containing the resin-bound peptide was washed with DMF (4 × 4 mL), $CH_2Cl_2$ (4 × 4 mL), $CH_3CN$ (2 × 4 mL), diethyl ether (2 × 4 mL), and $CH_2Cl_2$ (2 × 4 mL). For each individual wash, solvent was added, the vessel was capped and inverted twice, and then the solvent was removed by vacuum filtration.

**General Procedure B: Deprotection of an Allyl Ester Group.** The reaction vessel containing the resin-bound peptide was treated with $CH_2Cl_2$ (4 mL) and degassed by bubbling with argon for 5 min. *N*,*N*-Dimethylbarbituric acid (5 equiv) and tetrakis(triphenylphosphine)-palladium(0) (0.2 equiv) were added. The vessel was capped, agitated for 2 h, washed with diethyldithiocarbamic acid sodium salt (2% in DMF) (2 × 4 mL), and then washed according to General Procedure A.

**General Procedure C: Deprotection of an Fmoc Group.** To the reaction vessel containing the resin-bound peptide was added piperidine (20% in DMF) (4 mL). The vessel was capped, agitated for 20 min, and then washed according to General Procedure A.

**General Procedure D: Deprotection of a Trimethylsilylethyl (Tmse) Ester.** The reaction vessel containing the resin-bound peptide was treated with DMF (4 mL) and tetrabutylammonium fluoride (1 M in THF) (1.2 equiv). The vessel was capped, agitated for 24 h, and then washed according to General Procedure A. By microscale cleavage and

(25) Katoh, T.; Ueki, M. *Int. J. Pept. Protein Res.* **1993**, *42* (3), 264−269.

(26) Bergeron, R. J.; Wollenweber, M.; Wiegand, J. *J. Med. Chem.* **1994**, *37*, 2889−2895.

RP-HPLC analysis, it was found that two iterations of this procedure were required to affect complete deprotection.

**General Procedure E: Deprotection of an *o*-Nitrobenzenesulfonyl (Ns) Group.** A container of DMF was degassed for at least 30 min by bubbling argon through it. The reaction vessel containing the resin-bound peptide was treated with degassed DMF (4 mL), $\beta$-mercaptoethanol (20 equiv), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (10 equiv). The vessel was capped, agitated for 30 min, and then quickly washed with DMF (2 × 4 mL). By microscale cleavage and RP-HPLC analysis, it was found that six iterations of this procedure were required to affect complete deprotection. The beads were finally washed according to General Procedure A.

**General Procedure F: Coupling of Amino Acids using PyBOP**. To the reaction vessel containing the resin-bound peptide was added DMF/$CH_2Cl_2$ (1:1) (4 mL), the amino acid (3 equiv), 1-hydroxybenzotriazole (HOBt) hydrate (3 equiv), (*i*-Pr)2EtN (6 equiv), and benzotriazole-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (3 equiv). The vessel was capped, agitated for 15 min, and then washed according to General Procedure A.

**Solid-Phase Loading Sequence: Resin-Bound Intermediate 16.** To a solution of Fmoc-*cis*-Hyp-*O*-Allyl ester (**5**) (0.483 g, 1.23 mmol, 3 equiv) in 1,2-dichloroethane (5 mL) was added pyridinium *p*-toluenesulfonate (0.205 g, 0.817 mmol, 2 equiv) and 3 Å molecular sieves. The solution was briefly agitated to dry, decanted into a 25-mL round-bottom flask, and treated with 3,4-dihydro-2*H*-pyran-2-yl-methoxy methyl ("dihydropyranyl") polystyrene resin (**8**) (NovaBiochem part no. 01-64-0192; loading = 0.950 mmol/g) (0.430 g, 0.409 mmol, 1 equiv). The suspension was heated at 80 °C for 20 h, and then the resin was vacuum filtered through a Buchner funnel. The resin was then transferred into a Bio-rad Biospin 10-mL chromatography column ("reaction vessel") attached to a Bio-Rad 3-way stopcock, and the resin was washed (General Procedure A). The mass of the resulting dry resin was 0.515 g (53% loading); *thus for stoichiometry calculations in the remainder of the resin-bound steps, loading was assumed to be 0.217 mmol.*

The Hyp[4] allyl ester group was removed (General Procedure B), followed by the coupling of *H*-Ala-*O*-Tmse (**6**)[15] (General Procedure D). The coupling procedure was carried out twice to ensure complete reaction.

The Hyp[4] Fmoc group was removed by treatment with piperidine (General Procedure C), although for no more than 10 min because of concern of diketopiperazine formation. The chloranil test was performed to confirm the presence of free secondary amine.

Coupling conditions were adapted from Barany et al.,[20] to minimize racemization of cysteine. Thus, a solution of Trp[6]-Cys[3] fragment (7) (1.9 equiv) in DMF/$CH_2Cl_2$ (1 mL) was treated with 1-hydroxy-7-azabenzotriazole (HOAt) (2 equiv), 2,4,6-collidine (4 equiv), and (7-azabenzotriazole-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP) (2 equiv). The mixture was agitated by pipet for no more than 3−5 s and then quickly washed into the reaction vessel with DMF/$CH_2Cl_2$ (3 × 1 mL). The vessel was capped, agitated for 30 min, and then washed (General Procedure A). The chloranil test was negative, confirming absence of secondary amine and indicating complete coupling. Microscale cleavage and crude analysis by MALDI-TOF-MS were performed, revealing the presence of intermediate **16**.

**Resin-Bound Intermediate 4.** The Cys[3] Fmoc group was removed (General Procedure C, carried out twice for this one sluggish deprotection), and the resulting free amine was coupled with Fmoc-D-Thr(O-*t*-Bu)-OH (General Procedure F). This deprotection-coupling procedure was then repeated to attach Ala[1] and then Ala[7]. Microscale cleavage followed by separation using RP-HPLC and analysis by MALDI-TOF-MS showed the presence of intermediate 4 (Figures S3−S5; note that partial deprotection of *t*Bu was observed under the cleavage conditions).

**Resin-Bound Intermediate 3.** The Trp[6] allyl ester and Ala[7] Fmoc groups were removed (General Procedures B and C). The Kaiser test was positive, confirming the presence of free primary amine. To the reaction vessel containing the resin-bound peptide were added DMF/$CH_2Cl_2$ (1:1) (4 mL), 1-hydroxy-7-azabenzotriazole (HOAt) (3 equiv), (*i*-Pr)$_2$EtN (6 equiv), and (7-azabenzotriazole-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP) (3 equiv). The vessel was capped, agitated for 24 h, and then washed (General Procedure A). The Kaiser test was negative, confirming that the cyclization was complete. Microscale cleavage, followed by separation using RP-HPLC, and analysis by MALDI-TOF-MS showed the presence of intermediate **3** (Figures S6−S8; note that partial deprotection of Tmse was observed under the microscale cleavage conditions).

**[Ala[7]]-Phalloidin (1).** The Ala[5] trimethylsilylethyl (Tmse) and Trp[6] *o*-nitrobenzenesulfonyl (Ns) groups were removed (General Procedures D and E). The Kaiser test was performed, confirming the presence of free primary amine. Microscale cleavage followed by separation using RP-HPLC and analysis by ESI-MS showed the presence of the deprotected peptide (Figures S9 and S10).

To the reaction vessel containing the resin-bound peptide were added DMF (4 mL), (*i*-Pr)$_2$EtN (5 equiv), and diphenylphosphoryl azide (5 equiv). The vessel was capped, agitated for 48 h, and then washed according to General Procedure A. Microscale cleavage followed by crude analysis with MALDI-TOF-MS were performed, showing molecular weight consistent with [Ala[7]]-phalloidin (**1**).

The Bio-Rad 3-way stopcock was removed from the reaction vessel and replaced with a $CF_3CO_2H$-resistant polypropylene plug. A heterogeneous mixture of $CF_3CO_2H/H_2O/Et_3SiH$ (8:2:10 v/v/v) (6 mL) was prepared and added to the reaction vessel. The vessel was capped, agitated for 30 min, and then filtered into 100-mL recovery flask. The beads were then sequentially washed and filtered into the flask with $CH_2Cl_2$ (4 mL), $CH_3CN$ (4 mL), $CH_2Cl_2$ (4 mL), and $CH_3OH$ (2 × 4 mL). The resulting solution was concentrated in vacuo. Crude analysis by RP-HPLC (solvent gradient B) showed a mixture of compounds, including a ∼1:2 mixture of [Ala[7]]-phalloidin atropisomers, **1** (11.8 min) and **1′** (12.0 min) (Figure S10). RP-HPLC separation of **1** and **1′** was performed (solvent gradient B) to yield [Ala[7]]-phalloidin natural atropisomer **1** (5.7 min, 0.002 g, 1.3% yield based on initial loading **8**) and non-natural atropisomer **1′** (6.0 min, 0.005 g, 3.2% yield).

**[Ala[7]]-Phalloidin Natural Atropisomer (1).** $^1$H and $^{13}$C NMR (800 MHz, DMSO-$d_6$, 298 K): summarized in Tables S1 and S2. UV ($CH_3CN/H_2O$): $\lambda_{max}$ 291. CD ($CH_3OH$, 20 °C): positive Cotton effects 250, 305 nm. HRMS calcd for $C_{32}H_{42}N_8O_9S$ [M + Na] 737.2693, found [M + Na] 737.2681. (See Figures S12−S14.)

**[Ala[7]]-Phalloidin Non-Natural Atropisomer (1′).** $^1$H NMR (800 MHz, DMSO-$d_6$, 298 K): summarized in Tables S3 and S4. UV ($CH_3CN/H_2O$): $\lambda_{max}$ 295. CD ($CH_3OH$, 20 °C): negative Cotton effects 250, 305 nm. HRMS calcd for $C_{32}H_{42}N_8O_9S$ [M + Na] 737.2693, found [M + Na] 737.2708. (See Figures S15−S17.)

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**Supporting Information Available:** Supporting synthetic procedures for **5** and **12**; NMR spectra for **5**, **7**, **13**, **1**, **1′**; RP-HPLC chromatograms and MALDI-TOF/ESI-MS spectra for **15**, **16**, **4**, **3**, **1**; tabulated $^1$H and $^{13}$C NMR data; and computationally derived parameters for species **1** and **1′**. This material is available free of charge via the Internet at http://pubs.acs.org.

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