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Solid-phase synthesis of a novel phalloidin analog with on-bead and off-bead actin-binding activity†

Antoine Blanc, Mihajlo Todorovic and David M. Perrin *

Specific effectors of actin polymerization have found use as dynamic probes of cellular morphology that may be used to gauge cellular response to stimuli and drugs. Of various natural products that target actin, phalloidin is one of the most potent and selective inhibitors of actin depolymerization. Phalloidin and related members of the phallotoxin family are macrocyclic heptapeptides bearing a characteristic and rigidifying transannular tryptathionine bridge. Here we describe a solid-phase synthesis of a new phalloidin analog as a prototype for library development with the potential for on- and off-bead screening. To validate our method, we labelled the phalloidin derivative with a fluorescent dye which stained actin in CHO cells. Furthermore, a bioassay was developed allowing actin polymerization on beads carrying a phalloidin derivative.

The best-known and most specific ligand for F-actin is the natural product phalloidin (PHD), a bicyclic heptapeptide that belongs to a small class of structurally related peptides composing the phallotoxin family (Fig. 1).

Phallotoxins are biosynthesized from a ribosomally translated leader peptide that is cyclized and then further oxidized to yield the characteristic tryptathionine crosslink *inter alia*.[1] PHD binds non-covalently, selectively, and with high affinity (K_d < 40 nM) at the subunit interface of two actin monomers in F-actin to inhibit depolymerization, even in the presence of ions and proteins known to trigger F-actin depolymerization.[2] Given actin's central role in cellular homeostasis, it is often hijacked by numerous pathogens and is dysregulated in neoplastic tissue. Such events have inspired the design of actin-specific ligands as research tools and candidate therapeutics.[3] As such, fluorescently labelled PHD bioconjugates are widely used as specific probes of F-actin. With the exception of hepatocytes, the well-known poor cellular uptake of PHD explains its relatively low apparent cytotoxicity (IC_{50} >10 mM).[3b] Yet when PHD is conjugated to a targeting agent, toxicity against the targeted cells dramatically increases thus highlighting an opportunity to use PHD for precision therapeutics against cancer.[3b] Yet, the rational design of PHD analogs has proved challenging due to the absence of a high-resolution co-crystal structure of PHD bound to F-actin. To date, XPD studies along with biochemical studies, supported by a limited number of synthetic PHD derivatives, have implicated key structure–activity relationships (SARs).[4]

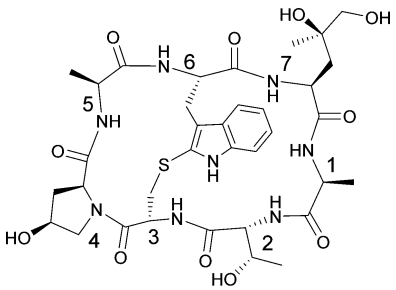

Fig. 1 Structure of phalloidin. Amino acid positions are numbered for reference.

A key feature of PHD is the transannular tryptathionine crosslink that provides essential rigidity while bisecting PHD into turns I and II. Turn I, (Cys[3]-*cis*-Hyp[4]-Ala[5]-Trp[6]), forms a rigid β-turn that is largely responsible for the binding interactions contributing to PHD toxicity. By contrast, turn II (Trp[6]-[γ, δ-di or γ-mono-hydroxy-Leu][7]-Ala[1]-[D-Thr/D-β-hydroxy-aspartic acid (D-Hya)][2]-Cys[3]) is relatively plastic and can accommodate various modifications as turn II is believed to extrude from the binding site.[4f,5] Wieland and co-workers pioneered the synthesis of phallotoxins; by exploiting the work of Savige and Fontana, they reacted *N*-α-protected 3*a*-hydroxyhexahydro-pyrrolo[2,3-*b*]indole-(2-carboxamide) (HPI),[6] with the S-Tr-cysteine in TFA to introduce the characteristic tryptathionine bridge. Alternate routes to PHD have been reported by Lokey and Guy.[7]

Despite these reports, PHD and its derivatives remain relatively unexplored, particularly in terms of combinatorial based

Chemistry Department, UBC, 2036 Main Mall, Vancouver, V6T-1Z1, Canada. E-mail: dperrin@chem.ubc.ca

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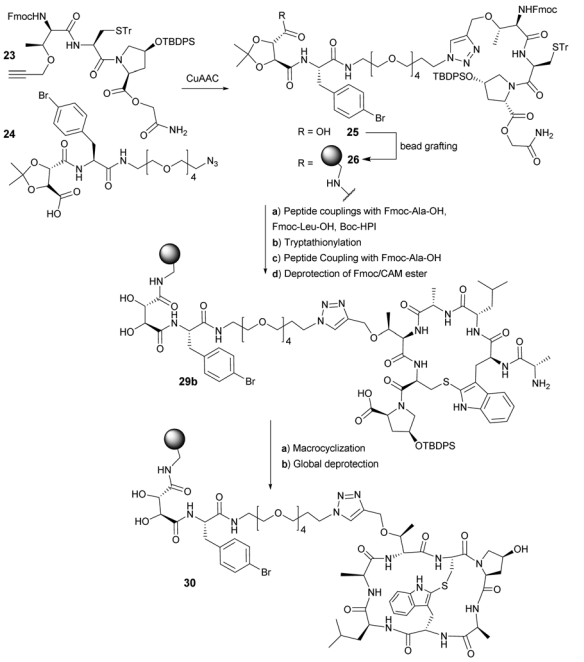
approaches. Hence, a long-term goal would be the discovery of new PHD analogues with specificity for certain pathognomonic actin isoforms using a One-Bead-One-Compound (OBOC) library, which in turn would expose new SARs of this venerated toxin.[8] More generally still, as there is interest in developing libraries of bicyclic peptide macrocycles, PHD could be a privileged scaffold for generating novel peptidic ligands. Towards these ends, here we report the Solid Phase Peptide Synthesis (SPPS) of an entirely synthetic PHD analogue to highlight the potential for on-bead and off-bead validation and inform the design of bicyclic heptapeptide libraries that may be used to target filamentous proteins as well as others.

To consider a synthesis of PHD with the potential for on-bead screening and off-bead validation, the PHD derivative would need to remain anchored to the bead throughout the synthesis yet be cleaved following. For this purpose, we sought a linker arm that would survive (i) the base treatment used in Fmoc deprotection and (ii) the relatively long (3–5 h) trifluoroacetic acid (TFA) treatment used to install the tryptathionine crosslink yet would be cleavable under mild conditions following synthesis. Hence, tartrate-based linker **6** was synthesized on TentaGel Macrobead (MB).[9] To assess whether this linker, once linked to PHD, would permit on-bead screening, an azide-linker was coupled with commercially available [Lys^7]-PHD and conjugated to **6** by a Copper(I)-catalyzed Cycloaddition (CuACC) as shown in Fig. 2 (see ESI† for details).[10] The beads linked to cpd **12** were then exposed to a solution of fluorescent G-actin. Following washing, the bead-bound F-actin was detected as a vividly fluorescent halo (Fig. 2) which was not observed in the presence of beads bearing only linker **6** (see ESI† for structure and full details).

Having validated the F-actin-stabilizing activity of a bead-bound PHD-bioconjugate, we considered a convergent SPPS route to provide a PHD analogue on the same beads. Although the success with **12** might have suggested grafting at position-7, we chose position-2 instead. Our choice to graft onto Thr^2 was primarily informed by a report that D-Thr^2 substitution by D-aminobutyric acid (D-Abu) did not significantly diminish PHD toxicity.[4c,e,11] In order to couple to a primary amine on the growing chain, we induced the tryptathionine crosslink immediately following HPI introduction thereby unmasking the N-terminal amine. This necessarily imposed directionality on an otherwise circularly permutable synthetic route that may be worthy of future consideration.[12] To minimize the potential for epimerization upon ring closure, we opted to macrocyclize between Hyp^4 and Ala^5 (Scheme 1), which necessitated the synthesis of a tripeptide with the sequence D-Thr-Cys-*cis*-Hyp.



Scheme 1 Solution phase CuAAC with linker (**24**) and tripeptide (**23**), followed by synthesis of [D-Thr^2(PEG-tartrate-TentaGel-MB), Leu^7]-PHD (**30**) on the solid phase.

With these considerations in mind, we approached the synthesis of the said PHD. The tripeptide N^{α}-Fmoc-O^{β}-propargyl-D-threonyl-S^{β}-trityl-cysteinyl-(1-carbamoylmethyl)-O^{γ}-(*tert*-butyldiphenylsilyl)-*cis*-hydroxyprolinate ester (**23**) was first synthesized (ESI†). Tripeptide **23** containing the Thr-*O*-propargyl ether was then conjugated to the azido tartrate-linker **24** *via* CuACC (Scheme 1).[13] We also added a Br-Phe into **24** to aid in eventual MS-identification of the final product, a feature found to be unnecessary for **12** that used linker **6**. Furthermore, we obtained a better yield by appending the alkyne functionality to the Thr of the tripeptide **23**; hence, an azide was introduced into the tartrate-based linker **24**.

Peptide-linker **25** was then loaded onto the TentaGel MB (ESI†) (Scheme 1) that afforded bidirectional synthetic capability.[14] The carboxamidomethyl ester (CAM) was chosen owing to its stability to both organic bases and to TFA.[15] The linear [D-Thr^2(PEG-tartrate-TentaGel-MB), Leu^7]-PHD heptapeptide precursor was synthesized by standard Fmoc/*tert*-Bu SPPS starting

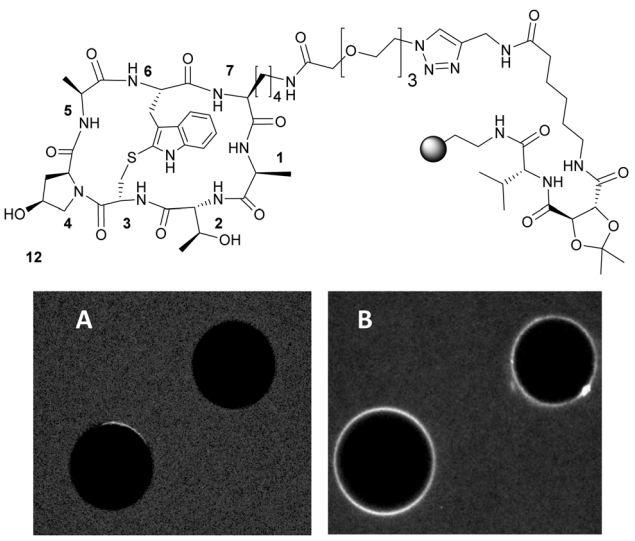


Fig. 2 Confocal image of beads (280–320 μm) with only linker **6** (negative control, for the structure of **6**, see ESI†) (Panel A) or linked to [Lys^7]-PHD (**12**) (Panel B). White rings correspond to 668 nm emission of F-actin polymerization, doped with actin that was labeled with Alexa Fluor 647 (545 nm excitation).

with tripeptide-linker resin **26** and terminating with the incorporation of N^{α}-Boc-HPI to yield hepta-peptide **27** on the solid support (Scheme 1 and ESI†). The N^{α}-Boc-3*a*-hydroxyhexahydro-pyrrolo-[2,3-*b*]indole-2-carboxylate (HPI) (**10**) was prepared as previously described and coupled to the resin-bound peptide. The resulting bead-bound hexapeptide (**27**) was then treated with TFA for 3–5 h to install the tryptathionine bridge.[6d] This unmasked the H_2N^{α} of the tryptathionine, which was then coupled to Fmoc-protected alanine. Following deprotection, a PyBOP-promoted macrocyclization gave the [D-Thr^2(PEG-tartrate-TentaGel-MB), Leu^7]-PHD (**30**) (Scheme 1). Each coupling step was effected with COMU in the presence of DMF-ethyl acetate-acetonitrile, followed by mild capping reaction.[16]

Subsequently, the TentaGel Macrobeads, linked to (**30**) were exposed to a solution allowing the polymerization of fluorescent G-actin to F-actin (ESI†). F-actin bound to bead-linked cpd **30**, was detected as a fluorescent ring (Fig. 3), albeit with diminished intensity compared to the case where the native PHD was directly linked to the beads. Such would be anticipated since the overall yield of PHD in this case is low, thus reducing the effective concentration of PHD on the bead (but not that of the linker arm or truncated/monocyclic) products.

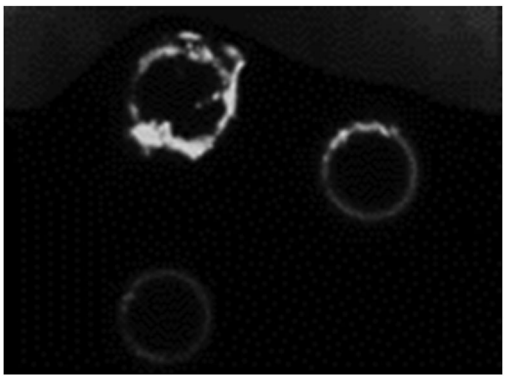
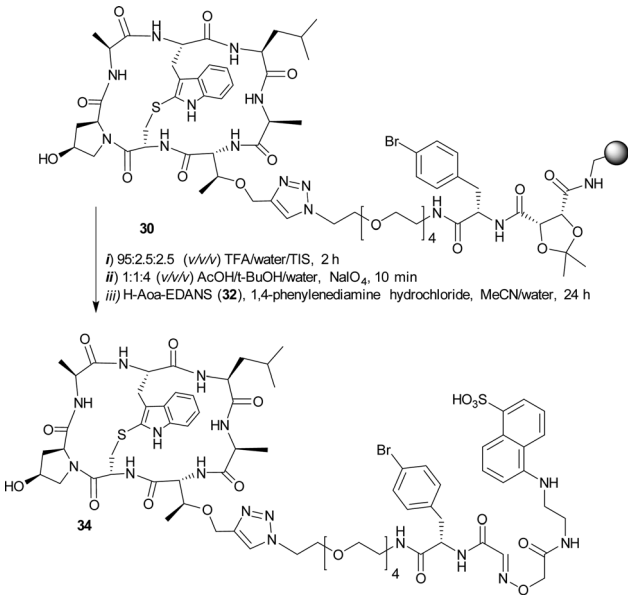


Fig. 3 Confocal image of beads (280–320 μm) linked to [D-Thr^2, Leu^7]-PHD (**30**) and probed using fluorescent actin as demonstrated in Fig. 2.



Scheme 2 Oxidative cleavage of the phalloidin derivative **30** and oximation with a fluorescent dye **32** to yield a fluorescent phalloidin conjugate **34**.

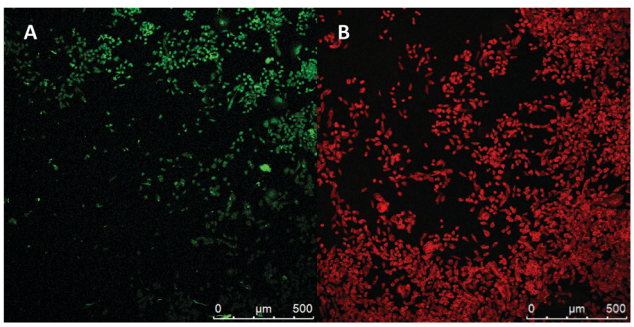


Fig. 4 Confocal microscopy (Obj 10×) of CHO cells exposed to fluorescent phalloidin (PHD) derivatives. Fixed and permeabilized CHO cells were incubated with [D-Thr^2(EDANS), Leu^7]-PHD (**34**) (Panel A, 405 nm excitation) or commercially available [HyLeu^7(TRITC)]-PHD (Panel B, 545 nm excitation).

To verify that the PHD on the bead was specific for actin, we cleaved the peptide from the bead and conjugated it to a fluorescent dye for a solution-phase actin staining assay. To do this, IO_4 treatment cleaved the diol, which had resulted from isopropylidene hydrolysis during the acid-catalyzed tryptathionylation reaction, to afford a glyoxamide handle. In a one-pot reaction, Boc-Aoa-EDANS (**31**) was deprotected and directly mixed under neutral conditions (pH 6–7) with the PHD glyoxamide derivative (**33**) to afford a new, fluorescently labelled [D-Thr^2(PEG-EDANS), Leu^7]-phalloidin (**34**) that was isolated with an unoptimized yield of 2–3% (Scheme 2 and ESI†).[17]

Following HPLC purification, the resulting [D-Thr^2(PEG-EDANS), Leu^7]-phalloidin (**34**) effectively stained Chinese hamster ovary (CHO) cells (Fig. 4) that had been permeabilized and fixed. In addition, no fluorescent signal was detected by fluorescent confocal microscopy when the CHO cells were pre-blocked with commercially available [HyLeu^7(TRITC)]-PHD thus demonstrating that staining by **34** is target-specific (for blocking experiment results, see ESI†).

Herein we prepared a synthetic PHD that can be assayed on- and off-beads by fluorescence. To demonstrate on-bead screening, we linked a commercially available PHD analog to the beads to demonstrate that a bead-bound PHD can elicit spatially defined F-actin polymerization. A major difference between the natural product and the synthetic PHD derivative **30** lies in the amino acid composition at position-2; in the bioactive natural product a non-alkylated D-Thr occupies this position. While the role that D-Thr plays in stabilizing F-actin remains ambiguous, our derivative corroborates past reports regarding a tolerance for D-Thr O^{β}-alkylation. As actin can be polymerized in a dynamic chain-reaction by various ligands,[18] it would be important to differentiate specific polymerization from non-specific aggregation that might be induced by intermediate products, the linker, or even the bead itself. Notably, no polymerization was observed

on beads attached to the linker. The lower yield of the fully synthetic PHD lowers the surface density on the bead, which in turn limits the F-actin polymerization seen for the synthetic PHD. It is thus unlikely that intermediate products and truncates gave the observed effect as monocyclic and *seco*-phalloidin analogs are inactive. To further corroborate bead-bound activity, the synthetic PHD was cleaved from the resin and derivatized to provide a fluorescent PHD-bioconjugate, which specifically stained the F-actin filaments in cells.

In contrast to previous solution- and solid-phase PHD syntheses that provide active analogs, here we demonstrate the potential for producing an active PHD analog where activity can be verified both on- and off-bead. These results now inform production of an OBOC library of PHD, which may find use in targeting various actin isoforms. Moreover, this work establishes a potential platform for creating libraries of other tryptathionine-bridged bicyclic peptides. While a late-stage tryptathionylation following macrocyclization[12] could be considered to enable a circularly permutable route thereby diversifying the starting amino acid and linkage site, such would require coupling to the sterically congested N^{α} of the HPI, which would lower yields. Such libraries can be screened on beads to identify new leads which could be readily bioconjugated. By employing the glyoxamide condensation chemistry here, one may readily link an antibody, aptamer, label, or toxin to a newly discovered bicyclic peptide lead. Based on these findings, bicyclic octapeptide libraries that could include amanitin analogs as inhibitors of transcription is also readily envisaged.

In summary, the salient points of this report are as follows: (1) a solid phase synthetic route towards a bead-bound synthetic phalloidin now incorporates linker-arm motifs for mass-spec tagging and biorthogonal bead-cleavage with the potential for fluorescent derivatization; (2) a bioassay allowing the specific F-actin polymerization on beads containing a phalloidin derivative has been performed; (3) these synthetic approaches inform strategies for the design of an OBOC array of a bicyclic peptide structure inspired by the phallotoxin family of peptides.

The synthetic procedures for named compounds and their spectroscopic data can be found in the ESI.†

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Conflicts of interest

There are no conflicts to declare.

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