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Authors: Roderich Süssmuth, Guiyang Yao, Jan-Oliver Joswig, and Bettina Keller

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Total synthesis of the death cap toxin phalloidin - atropoisomer-selectivity explained by molecular-dynamics simulations

Guiyang Yao,[a] Jan-Oliver Joswig,[b] Bettina G. Keller[b] and Roderich D. Süssmuth*[a]

Abstract: Phallotoxins and amatoxins are a group of prominent peptide toxins produced by the death cap mushroom *Amanita phalloides.* Phalloidin is a bicyclic cyclopeptide with an unusual tryptathionin thioether bridge. It is a potent stabilizer of filamentous actin and in a fluorescently labeled form widely used as a probe for actin binding. Herein, we report on the enantioselective synthesis of the key amino acid (2*S*,4*R*)-4,5-dihydroxy-leucine as a basis for the first total synthesis of phalloidin, which was accomplished by two different synthesis strategies. Molecular-dynamics simulations provided insights into the conformational flexibility of peptide intermediates of different reaction strategies and showed that this flexibility is critical for the formation of atropoisomers. By simulating the intermediates, rather than the final product, molecular-dynamics simulations will become a decisive tool in orchestrating the sequence of ring formation reactions of complex cyclic peptides.

In the past years peptides have received increased attention as potential drugs, since they could close a gap between small molecules and proteins:[1] They are smaller in size than proteins, but can address the inhibition of protein-protein interactions, while being commonly synthetically accessible and cheaper to manufacture. In this context, bioactive cyclic peptides constitute an excellent basis for studies directed towards the understanding of active and passive peptide transport and the design of peptides aiming for the delivery through membranes.[2] The cyclic peptide structure confers structural rigidity and thus bioactivity, while the absence of charges may facilitate transport. Prominent examples of that type of peptide from the group of fungal peptides are the amatoxins and the phallotoxins. Of the latter, phalloidin is the main representative.

Phalloidin **1** (Figure 1) is a bicyclic heptapeptide and its structure has been elucidated[3] and characterized by NMR spectroscopy studies[4] and the X-ray crystallographic analysis of [Ala]^7-phalloidin.[5] Characteristic structural features are one D-Thr and the unusual amino acids (2*S*,4*R*)-4,5-dihydroxy-L-leucine (DHLeu) and (2*S*,4*S*)-4-hydroxy-proline (Hyp). The latter is the less abundant *cis*-epimer, since commonly this post-translational hydroxylation, e.g. occurring in collagen, exclusively renders *trans*-4-hydroxyproline.[6] Finally, the phalloidin structure contains tryptathionin, a thioether amino acid constituting the bridge between the Cys sidechain and the 2-position of the indole ring of Trp.

Contrary to amatoxins, phalloidin is orally non-toxic. Its main toxicity (injected into the bloodstream) has been attributed to the uptake by the Organic Anion Transporting Polypeptide transporters OATP1P1[7] expressed on the sinusoidal side of hepatocytes.[8] Once taken up by the hepatocytes, its tight and selective binding to filamentous (F-)actin ultimately causes the destruction of the liver cells.[9] Modified derivatives of phalloidin containing fluorescent dyes are widely used to visualize F-actin for biomedical microscopy purposes.[10] A previous synthetic attempt toward [Ala]^7-phalloidin[11] however yielded two derivatives. It has been suggested that these derivatives are atropoisomers, i.e. isomers generated by the hindered rotation around the tryptathionin bridge,[5, 11] which places the indole sidechain of Trp either on the upper or the lower side of the plane of the macrolactam (Fig 2A and Fig 2B). However, further experimental details remained elusive.

Herein, we report on the total synthesis of phalloidin, which we envision as a biologically interesting and synthetically challenging molecule. Furthermore, our theoretical and experimental study aims for a rational and optimized synthesis strategy, as well as to explaining previously reported effects of atropoisomery. A robust total synthesis could further provide access to phalloidin derivatives for chemical biology studies, e.g. for the design of molecular probes. Finally, it opens the way to extended studies on structure-activity-relations, of transport phenomena across membranes, as well as provide options for further structural diversification.[12]

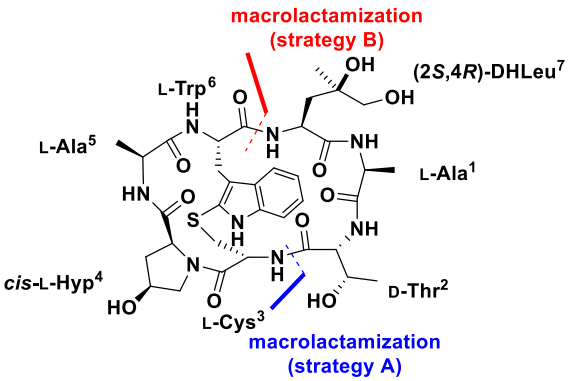

Figure 1. Structure of phalloidin (**1**) and strategic macrolactamization sites (strategy A and B) for the final ring closure; (2*S*,4*R*)-4,5-dihydroxy-L-leucine (DHLeu), (2*S*,4*S*)-4-hydroxy-proline (Hyp).

Key challenges of the synthesis were the access to a suitably protected (2*S*,4*R*)-4,5-dihydroxy-leucine (DHLeu) and the

[a] Dr. G. Yao, Prof. Dr. R. D. Süssmuth
Institut für Chemie, Technische Universität Berlin
Strasse des 17. Juni 124, 10623 Berlin (Germany)
E-mail: suessmuth@chem.tu-berlin.de

[b] J.-O. Joswig, Prof. Dr. B. G. Keller
Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Takustr. 3, 14195 Berlin (Germany).

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10.1002/chem.201901888

establishment of the tryptathionin bridge, while orchestrating an elegant sequence of ring formation reactions – if possible – avoiding atropoisomers. Two distinctive mechanisms for the formation of atropoisomers can be envisioned: (i) isomerization of natural phalloidin, or (ii) synthesis-based, during the assembly of the bicycle. We performed microsecond molecular-dynamics (MD) simulations of both phalloidin atropoisomers **1** and **1'** (Figure 2A and B). Neither at 300 K, nor at an elevated temperature of 500 K, spontaneous isomerization occurred. Furthermore, the peptide cycle formed multiple highly-populated intramolecular hydrogen bonds. Fully open ring structures were hardly ever sampled and had mean lifetimes of less than 2 ps. Especially, because the open ring structures are so short-lived, it seems very unlikely that spontaneous isomerization can occur even on timescales which are much larger than one microsecond. These results were confirmed by NMR spectroscopic investigations: HPLC-MS and NMR spectroscopic control of natural phalloidin **1** heated to elevated temperatures (90°C, DMSO) only led to signal shifts, but the compounds stayed intact (see Figure S7). Accordingly, the synthesis-based sequence of ring cyclization reactions must determine the occurrence of atropoisomers.

For the synthesis of the bicyclic structures of the phalloidin-type there exist two fundamentally different strategies: first the formation of the macrocycle followed by the I_2-mediated formation of the tryptathionin bridge [Glu]^7-phalloidin,[13] or the thioether formation prior to macrolactamization [Ala]^7-phalloidin.[11] The latter route is based on thioether formation of the Trp-Leu-Ala-D-Thr-Cys pentapeptide followed by attachment of a Pro-Ala dipeptide and macrolactamization ([5+2] strategy). Both approaches have limitations in applicability and the occurrence of by-products, e.g. unwanted dimerization and atropoisomers.

Which of the two atropoisomers is formed is determined by whether the ring formation occurs from above or from below the ring plane of the intermediate. In our MD simulations, we measured the orientation of the tryptathionin bridge with respect to the ring plane using the torsion angles indicated in Fig. 2C and 2D and compared to the corresponding distributions in phalloidin **1** and its atropoisomer **1'** (Fig. 2E). If the torsion angle is zero the tryptathionin bridge lies in the ring plane. The two atropoisomers are characterized by distributions on either side of the zero, corresponding to orientations of the tryptathionin bridge above and below the ring plane. Intermediate **a** contains the smaller and therefore less flexible β turn type I. Its torsion angle distribution coincides with this of natural phalloidin **1** and does not cross the zero line. The formation of **1'** is thus sterically impossible. By contrast, intermediate **b** contains the larger and therefore more conformationally flexible ring. Its torsion angle distribution crosses the zero line and shows overlap with the torsion angle distributions of both atropoisomers. Thus, ring formation from above and below the ring plane is possible for **b**. The synthesis strategy of [Glu]^7-phalloidin also yields selectively the natural atropoisomer, which is in line with our MD simulation of the intermediate of this strategy (**c**). Although the tryptathionine bridge is not yet formed, the side chains of Cys^3 and Trp^6 stayed on the side of the macrocycle which leads to the natural atropoisomer (see Supporting Information).

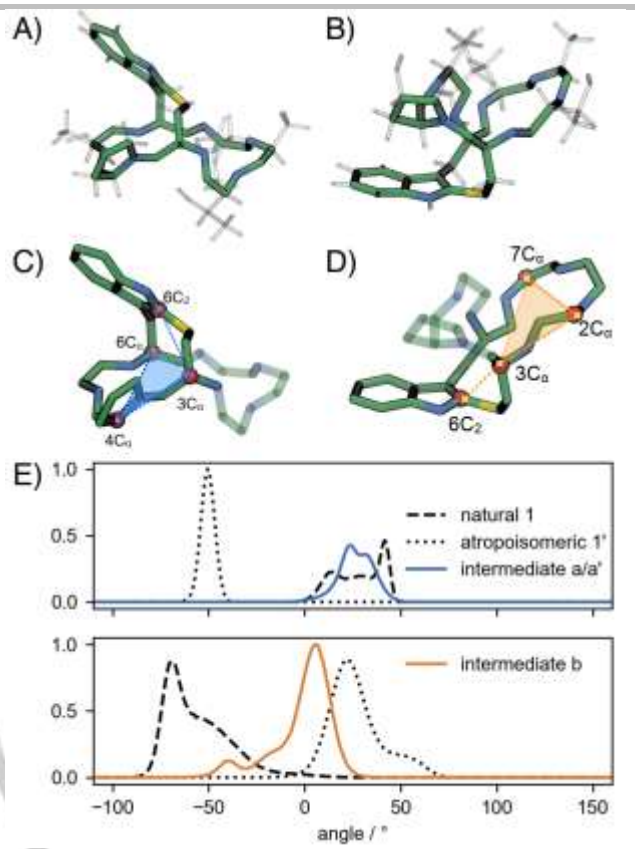

Figure 2: 3D structures of phalloidin **1** (A), its atropoisomer **1'** (B), ring structure of intermediate **a** or **a'** (C), ring structure of intermediate **b** (D). Peptide rings to be closed in subsequent synthesis steps are shown transparently in (C) and (D). The torsion angle of atom $6C_2$ with the ring plane is shown as a dotted line in (C) and (D). (E): Distribution of these two torsion angles in intermediate **a/a'** (blue) and intermediate **b** (orange) compared to the torsion angle distribution in **1** and **1'** (measured by MD simulations, see SI).

Based on these predictions from MD simulations, in our retrosynthetic analysis (Scheme 1), two strategies with final macrolactamization sites were chosen: between Cys^3 and D-Thr^2 or Trp^6 and DHLeu^7, respectively. In the first approach ([4+3]-strategy), we envisioned the assembly of the linear tetrapeptide cyclized by I_2-mediated tryptathionin formation to thioether **5** (β turn type I), which was then followed by coupling of a tripeptide **3** and macrolactamization to phalloidin (**1a**). In the alternative route (heptapeptide strategy), linear heptapeptide **19** (see Scheme 4) was synthesized on solid support, followed by the I_2-mediated tryptathionin formation, cleavage from the resin (monocyclic peptide **7**) and final macrolactamization to yield phalloidin **1b**.

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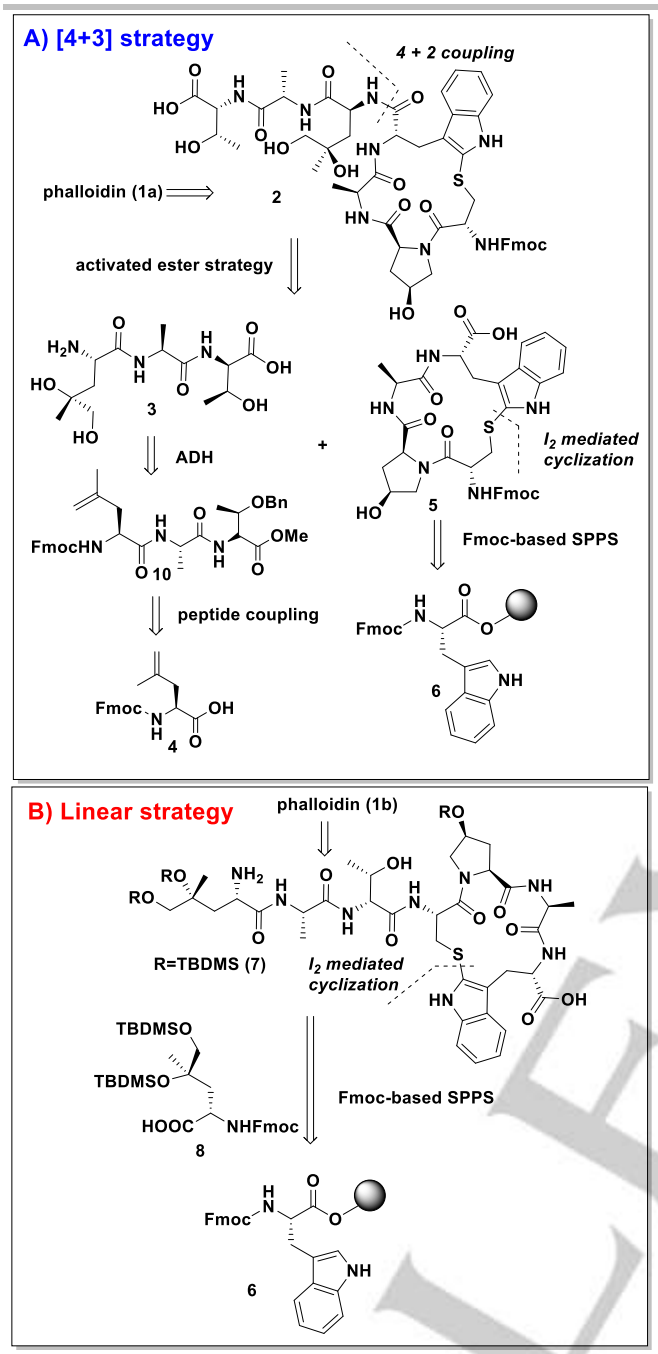
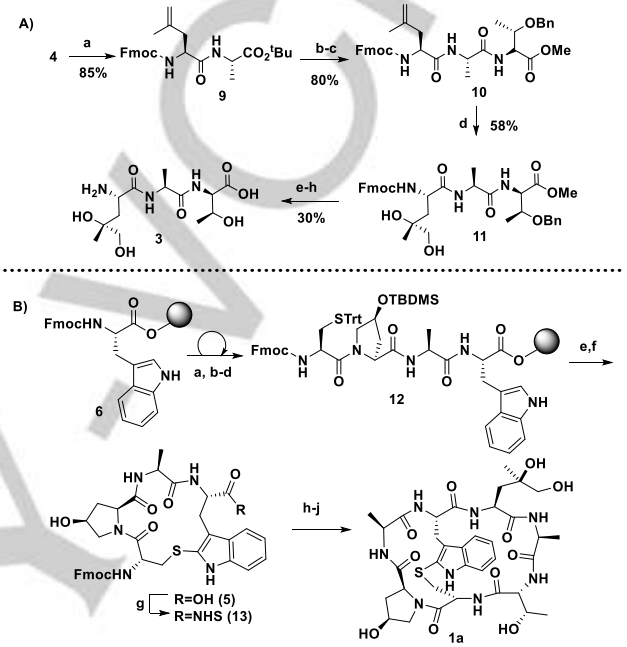


Scheme 1. Retrosynthetic analysis of phalloidin. A) [4+3]-strategy via monocyclic heptapeptide **2**; B) heptapeptide strategy from monocyclic peptide **7**, [Asymmetric Sharpless dihydroxylation (ADH)].

From the unusual amino acid building blocks of phalloidin, we had to consider *cis*-4-hydroxy-L-proline (4-Hyp) and (2*S*,4*R*)-4,5-DHLeu: Previously, 4-Hyp was reported to lactonize (4-OH group with the carboxy group) during peptide coupling.[14] Therefore, we prepared the TBDMS-protected *cis*-4-hydroxy-L-proline according to published protocols (see Supporting Information, Scheme S1).[15] A significant experimental challenge was a suitable synthetic approach to (2*S*,4*R*)-4,5-DHLeu: previous attempts as well as a recent chemoenzymatic route according to Renata et al.[16] yielded massive amounts of lactonized product, rendering these approaches futile. In order to prevent lactonization from esters or activation of the carboxy group of DHLeu, others employed a 4,5-didehydro-L-leucyl-L-alanine dipeptide. However, the subsequent Sharpless asymmetric dihydroxylation (ADH)[17] rendered inseparable mixtures of diastereomers. Our initial strategy with a 4,5-didehydro-L-leucine (Ddl)[18]-containing tripeptide (Fmoc-Ddl-Ala-Thr(Bzl)-OMe) **10**, (see Scheme 2A and Supporting Information) and subsequent Sharpless ADH rendered the desired peptide **11** with a diastereoselectivity (*dr* 4.8:1), which was transformed into tripeptide **3** for the subsequent attachment to monocyclic tetrapeptide **5**.



Scheme 2: Synthesis of phalloidin **1a** ([4+3]-strategy). A) Synthesis of tripeptide **3**, a) H-L-Ala-*O*^{t}Bu, HATU, DIPEA, DMF; b) TFA/DCM (1:1), 1h; c) H-D-Thr(*O*Bn)-*O*Me HATU, DIPEA, DMF; d) AD-mix-α, ^{t}BuOH/H_2O, 0°C, 12 h, 58% for the correct diastereomer after silica column; e) Et_2NH/MeCN (1:1), 15 min; f) Cbz-Cl, DIPEA, DMF, 5 h; g) 0.5 N LiOH/MeOH/THF (1:1:1), 2 h, then acidified by AcOH; h) H_2 (1 atm), Pd-C 10%, MeOH. B) Application of the [4+3]-strategy. a) piperdine/DMF (1:4); b) Fmoc-L-Ala-OH, TBTU, DIPEA, DMF; c) Fmoc-*cis*-4-L-Hyp(*O*TBDMS)-OH, TBTU, DIPEA, DMF; d) Fmoc-L-Cys(Trt)-OH, TBTU, DIPEA, DMF; e) 2 mg/ml I_2 in DMF, 2.5 h; f) TFA/TIS/H_2O (95:2.5:2.5); g) DCC, *N*-hydroxysuccinimide (NHS), DMF, 12 h; h) tripeptide **3**, DIPEA, DMF, 6 h; i) Et_2NH/MeCN, 15 min; j) EDCI (5 eq), HOAt (5 eq), DIPEA (20 eq), DCM/DMF (peptide concentration, 0.05 mM).

However, in order to enable a greater flexibility of the overall synthesis, we pursued the synthesis of an appropriately protected amino acid building block: The synthesis of protected enantiomerically pure (2*S*,4*R*)-Fmoc-4,5-DHLeu(TMDMS)$_2$-OH **8** (Scheme 3) was ultimately accomplished starting from methacryloyl chloride **14**, which was transformed into a Weinreb amide. After Sharpless ADH and silylation of the diol group with TBDMS, compound **15** was obtained in 65% yield with 90% ee. Subsequent to reduction with $LiAlH_4$, aldehyde[19] **16** was used in a Horner-Wadsworth olefination to form 2,3-didehydroamino acid ester **17**.[20] The protected dihydroxy-Leu derivative **17** was stereoselectively hydrogenated using with $Ru(COD)_2BF_4$ and (*R*)-MonoPhos as a ligand.[21] The hydrolysis of the methyl ester, hydrogenolytic removal of the Cbz-group and protection with Fmoc, rendered compound **8** in gram scale in 25% total yield. The stereochemistry of **8** was determined as (2*S*,4*R*) by acid-

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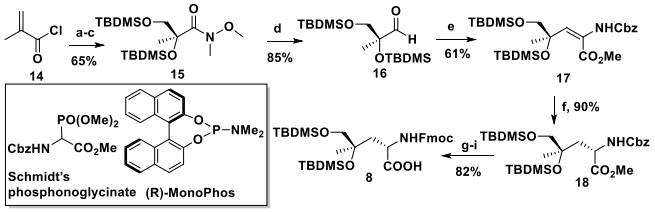
mediated lactonization to **S6** and compared to NMR spectra (see Figure S6).

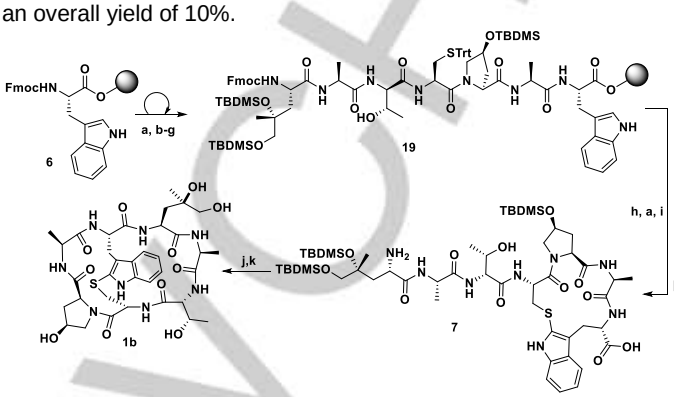


Scheme 3: Synthesis of protected DHLeu derivative **8**. a) *N,O*-dimethylhydroxylamine hydrochloride, Et_3N, DCM, 0 °C; b) AD-mix-β, $^tBuOH/H_2O$, 0°C; c) TBDMSCl, imidazole, DMF, 35 °C, 12 h; d) $LiAlH_4$, THF, -78°C, 1h, then aq 2M NaOH; e) Schmidt´s phosphonoglycinate, DBU, DCM, -20°C to rt, 16 h; f) H_2 (20 bar), 4mol % (R)-MonoPhos, 2mol% $[Ru(COD)_2BF_4]$, DCM, 4 h, rt; g) 0.5N LiOH/MeOH/THF (1:1:1), 2 h; h) H_2 (1 atm), Pd-C 10%, MeOH; i) Fmoc-OSu, $NaHCO_3$, 1,4-dioxane/H_2O, 12 h.

Thus equipped with the required building blocks, we focussed on peptide assembly and the establishment of the tryptathionine bridge (Scheme 1). Although the Savige-Fontana reaction has been wildly used for formation of the thioether peptide, including the first total synthesis of α-amanitin, amatoxins as well as phallotoxins,[22] the pre-oxidation of Trp yielding 3a-hydroxypyrrolo [2,3-b]indoline (Hpi) and the subsequent reaction with Cys involves a considerable number of steps. We decided to use direct thionation of a non-protected Trp sidechain and trityl-protected Cys by iodine oxidation, a mild an efficient reaction for thioether coupling.[13, 23]

According to our [4+3] strategy via the thioether tetrapeptide **5**, we performed the assembly of linear precursor peptide **12** on 2-chlorotrityl (2-CTC) resin by Fmoc-solid phase peptide synthesis (SPPS) starting with the immobilization of unprotected Fmoc-Trp-OH (see Supporting Information). A resin loading of 0.30 mmol/g was preferred over higher loadings, which could promote unwanted intramolecular tryptathionine formation. Tetrapeptide **5** (Scheme 2) was synthesized according to a sequence of amino acid couplings and Fmoc-deprotection cycles: Fmoc-Ala-OH, Fmoc-*cis*-4-Hyp(*O*TBDMS)-OH and Fmoc-Cys(Trt)-OH, followed by I_2-mediated cyclization. Upon cleavage from the resin with TFA/TIS/H_2O (95:2.5:2.5), and subsequent preparative HPLC purification, monocyclic tetrapeptide **5** was obtained in an overall yield of 47%. Then, we turned to coupling of **5** and tripeptide **3** using hydroxysuccinimide (NHS) ester-mediated coupling conditions (DCC), which proceeded smoothly to yield heptapeptide **2**. After removal of the Fmoc protecting group, macrocyclization was performed with EDCI/HOAt/DIPEA. HPLC purification yielded synthetic phalloidin (**1a**) which was NMR spectroscopically characterized and found to be identical with natural phalloidin **1** (see Supporting Information).

Then we assessed the alternative SPPS-based route from a linear heptapeptide: Fmoc-based synthesis proceeded smoothly employing TBTU as the coupling reagent in the sequence (Scheme 4, see also Supporting Information): Fmoc-L-Ala-OH, Fmoc-*cis*-L-Hyp(OTBDMS)-OH and Fmoc-L-Cys(Trt)-OH. Fmoc-D-Thr-OH with free hydroxy group was coupled using HATU/HOAt, followed by coupling of Fmoc-L-Ala-OH and Fmoc-DHLeu$(TMDMS)_2$-OH **8** using TBTU as a coupling reagent. According to the protocol mentioned above, resin-bound peptide **19** was treated with I_2 in DMF for 2.5 h.[13] Cleavage from the resin of monocyclic thioether-peptide **7** was performed with TFE/HOAc/DCM (1:1:8) and preparative HPLC purification rendered monocyclic peptide **7** in 30% yield. The final macrolactamization to phalloidin was most favorable for HATU/collidine in DCM/DMF (5:1) as a coupling reagent. After removal of the silyl protecting groups from the hydroxy functions of DHLeu with TBAF/THF, the crude peptide was purified by reversed-phase HPLC. Synthetic phalloidin **1b** was obtained in an overall yield of 10%.



Scheme 4: Synthesis of phalloidin **1b** from a linear heptapeptide precursor. a) piperdine/DMF (1:4); b) Fmoc-L-Ala-OH, TBTU, DIPEA, DMF; c) Fmoc-*cis*-4-L-Hyp(OTBDMS)-OH, TBTU, DIPEA, DMF; d) Fmoc-L-Cys(Trt)-OH, TBTU, DIPEA, DMF; e) Fmoc-D-Thr-OH, HATU, HOAt, DIPEA, DMF; f) Fmoc-L-Ala-OH, TBTU, DIPEA, DMF; g) **8**, TBTU, DIPEA, DMF; h) 2 mg/ml I_2 in DMF, 2.5 h; i) TFE (trifluoroethanol)/HOAc/DCM, (1:1:8); j) HATU (5 eq), collidine (20 eq), DCM/DMF (peptide concentration, 0.05 mM), 12 h; k) 1M TBAF in THF, 30 min.

Notably, with our coupling strategies no atropoisomers were observed unlike previously reported for the [5+2]-strategy.[10] The circular dichroism (CD) spectra of synthetic phalloidins **1a/1b** (Figure 3) and the ^1H NMR spectra were consistent with those of natural phalloidin **1** (see Figure S1). Likewise, the calculated CD spectra of **1** are in very good agreement with the experimental spectra. By contrast, the calculated CD spectrum for atropoisomer **1'** differs clearly from the experimental and calculated spectra of **1**, in particular in the critical region of λ = 240 to 260 nm, in which the two spectra have different signs (see SI).

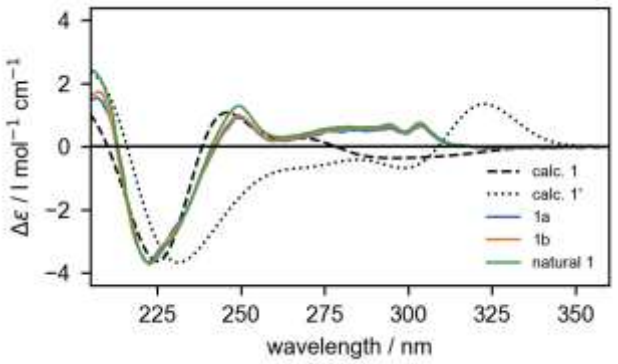


Figure 3: Circular dichroism spectra of synthetic phalloidin (**1a** and **1b**), natural phalloidin (**1**) as well as simulation spectra of phalloidin **1** and atropoisomer **1'**.

In summary, we herein present the first total synthesis of the multicyclic peptide toxin phalloidin, also giving access to an enantioselective synthesis of (2*S*,4*R*)-4,5-dihydroxy-L-leucine (DHLeu). The synthetic strategy to phalloidin is based on MD

calculations which guided the way for the identification of suited macrocyclization sites in an exclusive favor of the correct natural atropoisomer. Furthermore, our study excludes the natural occurrence of atropoisomery by a ring flip, and rather gives an explanation for the occurrence of atropoisomery during synthesis promoted by the greater conformational flexibility of the pentapeptide ring.

The study shows that, for devising synthesis strategies of complex and (multi)cyclic molecules, it is essential to have precise knowledge of the conformational flexibility of all intermediates - preferably already at the stage of a retrosynthetic analysis. Molecular dynamics simulations provide this information and have evolved into a tool, which can be used on a routine basis. Thus far, molecular dynamics studies have focused on the final peptides. We believe that by simulating the intermediates rather than the final product, molecular dynamics simulations will become a decisive step in the prior assessment of synthetic methods. The synthesis will further facilitate the synthesis of new phalloidin derivatives and structure activity-relationship studies.

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Keywords: Phalloidin • *Amanitia phalloides* • cyclopeptide • tryptathionine • thioether • molecular dynamics simulations

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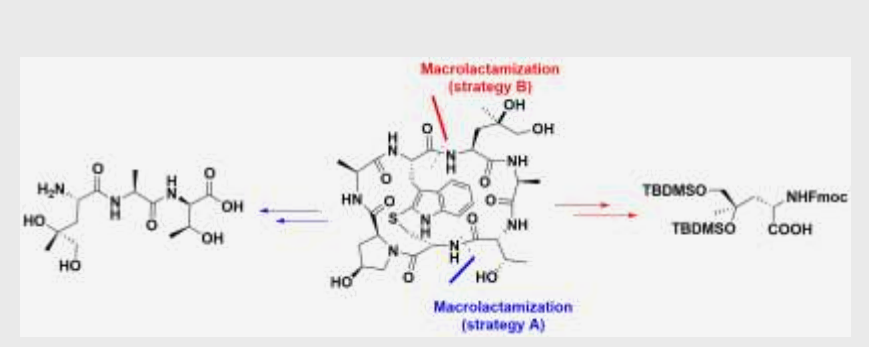

*Guiyang Yao, Jan-Oliver Joswig, Bettina G. Keller and Roderich D. Süssmuth**

Page No. – Page No.

Total synthesis of the death cap toxin phalloidin - atropoisomer-selectivity explained by molecular-dynamics simulations

Molecular-dynamics simulations guide ring formation: The first total synthesis of phalloidin was achieved through the enantioselective synthesis of (2*S*, 4*R*)-4,5-dihydroxy-leucine, which was accomplished by two different synthesis strategies. Molecular-dynamics simulations showed that this flexibility of intermediates is critical for the formation of atropoisomers and will become a decisive tool in orchestrating the sequence of ring formation reactions of complex cyclic peptides.