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Total Synthesis of Alloviroidin

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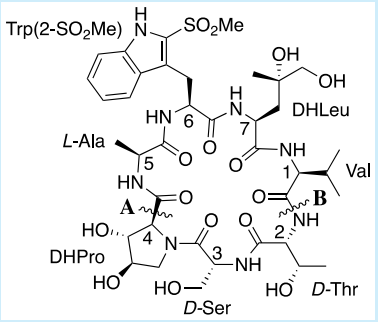
Supporting Information

ABSTRACT: Alloviroidin is a cyclic heptapeptide, produced by several species of *Amanita* mushrooms, that demonstrates high affinity for F-actin as is characteristic of virotoxins and phallotoxins. Alloviroidin was synthesized via a [3 + 4] fragment condensation of Fmoc-D-Thr(OTBS)-D-Ser(OTBS)-(2*S*,3*R*,4*R*)-DHPro(OTBS)$_2$-OH and H-Ala-Trp(2-SO$_2$Me)-(2*S*,4*S*)-DHLeu(5-OTBS)-Val-OMe to form bond **A**. The linear heptapeptide favored a turn conformation, facilitating cyclization between Val[1] and D-Thr[2] (position **B**). Global deprotection and HPLC purification afforded alloviroidin with NMR spectra in excellent agreement with the natural product.



Toxins of the *Amanita* mushrooms have been the subject of folklore and murder mysteries. Fascination with these notorious compounds and their pharmacology was the life's work of Theodore Wieland.[1] Ongoing interest and developments have recently been updated in Walton's monograph.[2] Chemical synthesis, exemplified by an elegant approach to amanatoxin by Perrin's group last year,[3] is challenging due to the density of post-translational modifications and bridging functionality. One of the best-known toxins is the bicyclic heptapeptide, phalloidin (**1**), characterized by a thioindolyl bridge, which adopts a rigid conformation. This protypical phallotoxin binds tightly to F-actin and is the ongoing target of synthetic chemistry.[4] Conjugated to fluorophores, phalloidin is widely used to study the dynamics of actin filaments.[5] Modulators of actin polymerization are valuable in the study of the cytoskeleton of cancer cells,[6] a target for chemotherapeutic drugs.[7] The virotoxins (e.g., **2** and **3**, Figure 1) are recognized members of the cast of amanotoxins but have been less well-studied. They were first isolated from *Amanita virosa*[8]—the so-called "destroying angel"—and have also been identified in extracts from *A. suballiacea*[9] and *A. subpallidorosea*.[10] With respect to white mice, the LD$_{50}$ of alloviroidin is 1−2 nmol/g when administered via intraperitoneal injection.[9b] Virotoxins have the same rigid conformation[11] as the phallotoxins and bind actin with comparable affinity.[12] This is quite remarkable given the monocyclic nature of the compounds. The rigid conformation is likely due to the amino acids in positions 3 and 4, a D-Ser[13] and a (2*S*,3*R*,4*R*)-3,4-dihydroxy-L-proline (DHPro),[14] respectively.

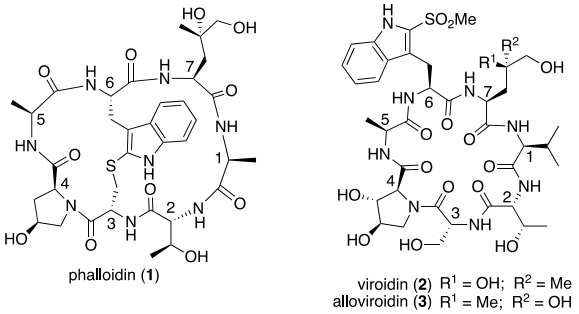


Figure 1. Selected *Amanita* toxins.

Virotoxin analogs have been synthesized by Wieland and coworkers, substituting *cis*-4-hydroxy-L-proline at position 4.[15] Further structure−activity studies have been limited by the availability of the unusual constituent amino acids. Several years ago we developed a reliable route to 3,4-dihydroxyprolines from pentose sugars.[16] In 2009, we reported the synthesis of a dipeptide containing a (2*S*,4*S*)-4,5-dihydroxyleucine (DHLeu) residue,[17] as found in alloviroidin. We have also studied the impact of prolyl hydroxylation on peptide conformation.[18] Thus, we were uniquely positioned to contemplate the synthesis of virotoxins.

In a landmark discovery, about 10 years ago, Hallen et al. demonstrated that amanatoxins and phallacins, produced by *A. bisporangia* (another destroying angel), were the first known example of cyclic peptides to be produced by ribosomal peptide synthesis in a fungus.[19] The variable toxin region of the linear precursor peptides is converted to cycloamanides (cyclic peptides from *Amanita* species) in two steps, catalyzed by a specialized prolyl oligopeptidase (POPB).[20]

While biosynthesis is often an inspiration for chemical synthesis, that is rarely so in peptide chemistry. Fundamentally, ribosomal peptide synthesis proceeds from N- to C-terminus, whereas chemical synthesis is routinely conducted in the reverse direction to avoid epimerization of Cα stereogenic centers. A C-terminal Pro residue is advantageous in peptide fragment condensations, because Pro is not susceptible to epimerization.

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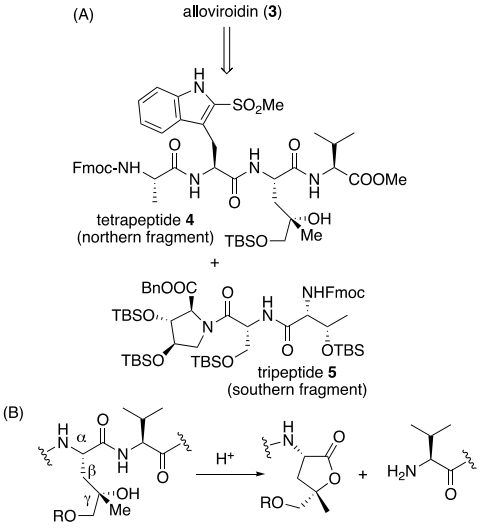
However, to facilitate cyclization, we advocate for embedding the Pro residue in the middle of the linear peptide, in the expectation that there will be conformations through which cyclization is entropically enabled. Indeed, others have invoked "pseudoprolines" to facilite the cyclization of recalcitrant sequences.[21] Thus, our retrosythetic analysis is presented in Scheme 1A. Amalgamation of a "northern" tetrapeptide amine

Scheme 1. (A) Retrosynthetic Analysis of Alloviroidin (3) and (B) Backbone Cleavage of Peptides Containing γ-Hydroxy-amino acids under Acidic Conditions



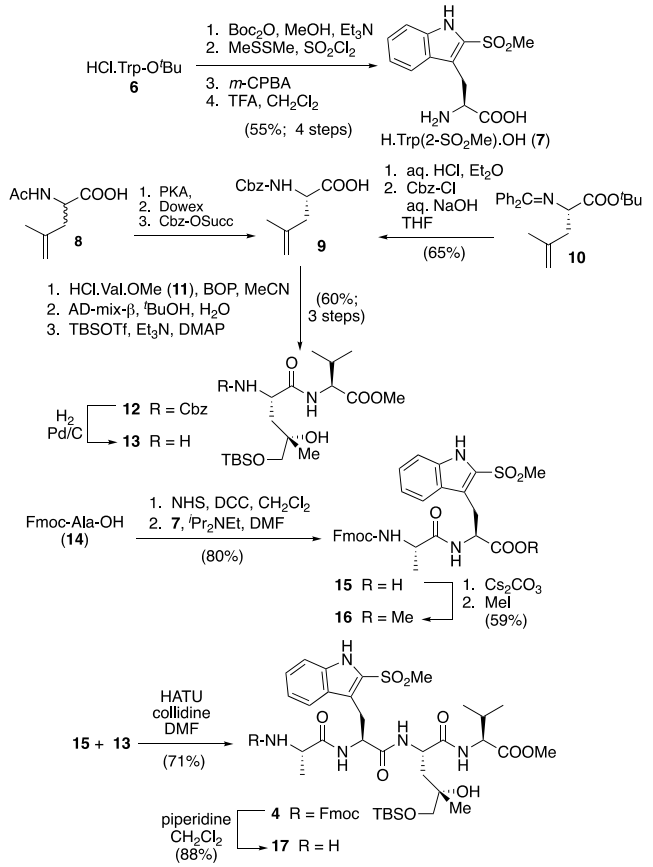
(derived from **4**) and a "southern" tripeptide acid (derived from **5**) would generate a linear heptapeptide with the DHPro residue in the third position. This was appealing, since the activated tripeptide, with its *C*-terminal dihydroxyproline residue, would not be susceptible to epimerization during coupling/cyclization (vide infra). This penultimate step should also be favored by the fact that the two residues (L-Val and D-Thr) are of opposite configuration,[22] albeit somewhat hindered.

While modern peptide synthesis is dominated by Fmoc chemistry, in conjunction with acid-labile side chain protecting groups, this approach would have been incompatible with the DHLeu residue. Under acidic conditions, γ-hydroxyamides form γ-lactones with concomitant cleavage of the amide bond[17] (Scheme 1B). We elected to employ fluoride-labile TBS ethers[23] as protecting groups for the heavily hydroxylated peptide. The application of silyl ethers in peptide chemistry has gathered momentum in the past decade,[3,24,25] notably in the presence of functional groups that do not tolerate acidic conditions, e.g., the recent synthesis of imine-containing scytonemide A by Wilson et al.[26]

Construction of the 2-methylsulfonyl-tryptophan residue [Trp(SO_2Me)] is depicted in Scheme 2. Specifically, Boc-Trp-O^{t}Bu was subjected to methylsulfenyl chloride generated *in situ*.[27] While indoles typically undergo S_EAr at C-3, it is well established that tryptophan derivatives react with alkylsulfenyl chlorides to afford the 2-thioether,[28] presumably via initial attack at C-3 followed by rearrangement.[29] The resulting thioether was not particularly stable, so it was oxidized immediately to the corresponding sulfone. Liberation of the free amino acid **7** was accomplished under acidic conditions.

Our original synthesis of (2*S*,4*S*)-4,5-dihydroxyleucine (DHLeu) included resolution of *N*-acetyl-dehydroleucine

Scheme 2. Synthesis of Tetrapeptide 4



(Dhl) (**8**) by porcine kidney acylase (PKA), Cbz-protection, and incorporation into a dipeptide followed by a Sharpless asymmetric dihydroxylation of the side chain double bond (Scheme 2). On the basis of our subsequent experience[30] with the Corey–Lygo asymmetric synthesis of amino acids,[31] we recognized that the synthesis of Dhl was an ideal application of this method, since the benzophenone imine of glycine *tert*-butyl ester would be alkylated by an allylic electrophile. Known compound **10**[31a] was readily converted to Cbz-Dhl-OH (**9**) and condensed with valine methyl ester (**11**). Elaboration, as described previously,[17] gave dipeptide **12**. Activation of Fmoc-Ala-OH (**14**) and condensation with tryptophan **7** gave dipeptide acid **15**; for the purposes of characterization this was converted to methyl ester **16**. Hydrogenolysis of the *N*-terminal Cbz group of compound **12** gave amine **13** that was coupled with acid **15**, according to Carpino,[32] to give tetrapeptide **4** in good yield, with no epimerization of the Trp residue observed. The tetrapeptide fragment was stored in the form of **4** and deprotected immediately prior to the fragment condensation.

The synthesis of tripeptide **5** is summarized in Scheme 3. (2*S*,3*R*,4*R*)-3,4-Dihydroxy-L-proline (DHPro)[14] building block **18** was prepared as described previously.[16a] Dipeptide acid **23** was prepared in the standard manner (*vide supra*) and coupled with dihydroxyprolylamine **20**. As discussed earlier, we needed to substitute for the acid-labile protecting groups. Removal of these protecting groups, notably the MEM ethers, was not straightforward. Cleavage with TFA in dichloromethane was sluggish; treatment with $TiCl_4$[33] sometimes gave good results, but was not reproducible. Ultimately, treatment with hydrochloric acid in TFA[34] was the most effective and reliable.

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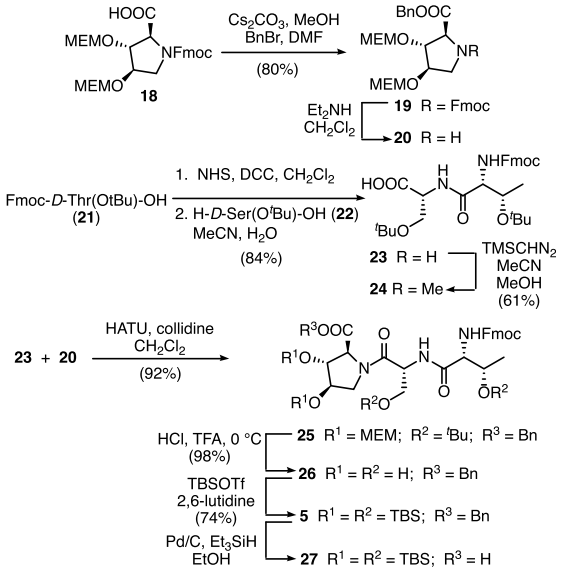
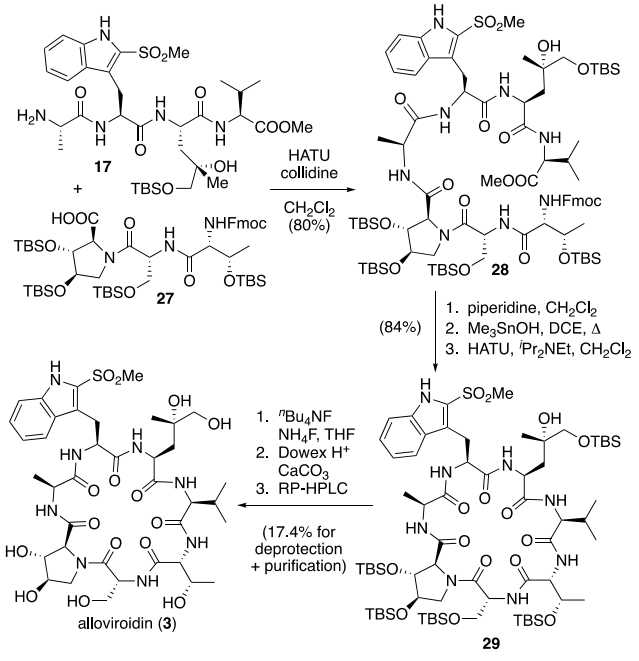
Scheme 3. Synthesis of Tripeptide 5



Protection of the four alcohols as TBS ethers was uneventful. The *C*-terminal benzyl ester in **5** was removed hydrogenolytically to afford acid **27**. This reaction had to be monitored carefully, as cleavage of the Fmoc group was observed on prolonged exposure; triethylsilane was preferable to hydrogen gas in this regard.

Coupling of tripeptide acid **27** and tetrapeptide amine **17** gave linear heptapeptide **28** in good yield using HATU/collidine (Scheme 4). The N-terminus was deprotected by treatment with piperidine; it was advantageous to purify the heptapeptide amine by flash chromatography at this stage. The C-terminal methyl ester was hydrolyzed with trimethyltin hydroxide, using the conditions initially reported by Nicolaou et al.[35] The linear heptapeptide with free termini was extracted from pH 5.5

Scheme 4. Fragment Condensation, Cyclization, Deprotection, and Purification To Afford Alloviroidin (3)

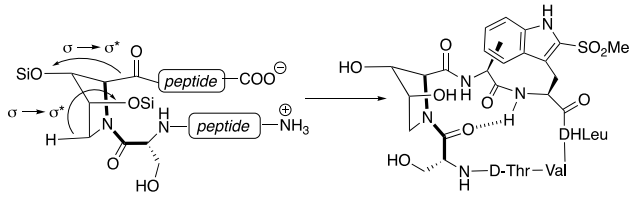


phosphate buffer with ethyl acetate and used without further purification. Cyclization was achieved with HATU. Use of collidine as base was not as effective as diisopropylethylamine. Cyclizations were originally attempted in DMF, but found to be faster and cleaner in dichloromethane, at a concentration of 1 mM, affording an excellent yield of cyclic heptapeptide **29**.

On standing, partial cleavage of one or more TBS ethers from **29** was observed, making it impossible to obtain good NMR data. Thus, we proceeded directly with a global deprotection using buffered tetrabutylammonium fluoride.[36] DiLauro et al. recently demonstrated that it is not necessary to use stoichiometric fluoride;[37] deprotection of the five TBS groups, to afford **3**, was accomplished with 2.5 equiv of $^{n}Bu_4NF$ overnight. We experienced great difficulty separating the resulting alloviroidin (**3**) from salts and found that the protocol of Kishi and Kaburagi[38] was beneficial. Reversed phase HPLC then afforded pure alloviroidin in disappointing yield, but high purity. Synthetic **3** was characterized by NMR in D_2O/H_2O at 500 MHz, with full assignment of signals (see Supporting Information). Proton NMR spectra acquired in DMSO-d_6 were in excellent agreement with those of the natural product (see Supporting Information).

Compounds **19**, **25**, **26**, and **5** existed as mixtures of conformations about the prolyl peptide bond. ^{1}H NMR indicated ratios ranging from 1:1 (for **19**) up to 2:1 (for **25**), presumably favoring the trans conformation. Since NMR spectra were complicated by these equilibria, we demonstrated the purity of key compounds by normal phase HPLC (see Supporting Information). Our earlier studies on the conformation of dipeptides containing hydroxylated prolines[18] did not include the (2*S*,3*R*,4*R*)-DHPro isomer. Crystallographic evidence is available for related monohydroxylated prolines with (2*S*,3*R*) [3-OH][39] and (2*S*,4*R*) [4-OH][40] configurations. Both amino acids demonstrated a C*β-exo* conformation of the pyrrolidine ring. In the latter case, the residue was incorporated into collagen-like sequences and found to destabilize the triple helix, implying a destabilization of the trans prolyl peptide bond relative to its cis rotamer. We suspect that the DHPro residue in the virotoxins also prefers a C*β-exo* conformation (Scheme 5)

Scheme 5. DHPro-Induced Turn in Linear Peptide Facilitates Cyclization Followed by "Relaxation" to Type II *β*-Turn



that is stabilized by hyperconjugative donation from σ(Cα-CO) → σ^*(Cβ-O) and σ(Cδ-H$_{ax}$) → σ^*(Cγ-O). The proton NMR signals for Hα, Hβ, and Hγ of peptides containing the DHPro appear as broad singlets, consistent with small coupling constants in predominantly the Cβ-exo conformation. Further, we speculate that a turn induced by the proline residue brings the two "arms" of the peptide close enough to enable cyclization. Following ring formation and deprotection, alloviroidin adopts a type II β-turn in this region of the molecule, with the prolyl peptide bond in the trans conformation, as previously described by Kobayashi et al., who noted shielding of the Ala methyl group by the aromatic side chain of the adjacent Trp residue and a hydrogen bond between the D-Ser C═O and the Trp NH.[11a]

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In summary, we have prepared alloviroidin from the seven constituent amino acids via a solution-based fragment condensation approach. There are two longest linear sequences for the peptide assembly, including deprotection steps and purification: 11 steps from DHPro **18** (7.7% overall) and 11 steps from DHLeu **9** (5.4% overall). To the best of our knowledge this represents the first total synthesis of a virotoxin natural product.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b00567.

Procedures for the synthesis of all compounds in Schemes 2−4, along with their ^{1}H and ^{13}C NMR spectra and HPLC traces for compounds **10**, **4**, **5**, and **28**; assigned $^{1}H-^{1}H$ COSY and HSQC spectra for alloviroidin (**3**), along with ^{1}H NMR comparisons of synthetic and natural material (PDF)

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Notes

The authors declare no competing financial interest.

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