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A Convergent Total Synthesis of the Death Cap Toxin α-Amanitin

Mary-Ann J. Siegert,[a],[‡] Caroline H. Knittel,[a],[‡] Roderich D. Süssmuth* [a]

This paper is dedicated to Dr. Gerhard Jas †

Abstract: The toxic bicyclic octapeptide α-amanitin is mostly found in different species of the mushroom genus *Amanita*, with the death cap (*Amanita phalloides*) as one of the most prominent members. Due to its high selective inhibition of RNA polymerase II which is directly linked to its high toxicity, particularly to hepatocytes, α-amanitin received an increased attention as a toxin-component of antibody-drug conjugates (ADC) in cancer research. Furthermore, the isolation of α-amanitin from mushrooms as the sole source severely restricts compound supply as well as further investigations, as structure-activity relationship (SAR) studies. Based on a straightforward access to the non-proteinogenic amino acid dihydroxyisoleucine we herein present a robust total synthesis of α-amanitin providing options for production at larger scale as well as future structural diversifications.

Amatoxins are ribosomally synthesized toxic bicyclic octapeptides mainly found in different species of the fungus genus *Amanita*, most notably *Amanita phalloides*.[1–4] These compounds are selective inhibitors of eukaryotic RNA polymerase II [α-amanitin, $K_d = 10^{-9}$ M], which ultimately leads to apoptosis.[4,5] The fungal toxins were first isolated by Heinrich Wieland and Rudolf Hallermayer in 1941 and their structure was elucidated in the 1950s and 1960s (Wieland *et al*.).[6,7] All amatoxins contain eight L-configured amino acids and a tryptathionine linkage between the side chains of tryptophan and cysteine, with an (*R*)-configured sulfoxide moiety.[1,3] However, the amanitins differ in their hydroxylation pattern of the amino acid side chains, which modulates their toxicity. The effect of the differently hydroxylated amino acids on the amatoxin toxicity was investigated in various studies.[8–11]

In the past 30 years several approaches were made to synthesize amatoxin derivatives.[8,12–15] Zanotti *et al*. (1987) described the first synthesis of an amaninamide derivative starting from the linear octapeptide followed by thioether formation using the Savige-Fontana reaction and subsequent macrocyclization.[8] The Savige-Fontana methodology is based on an *N*-terminal Hpi (3*a*-hydroxypyrollo[2,3-*b*]indole) moiety and a trityl side-chain protected cysteine residue. Simultaneous deprotection of the cysteine residue and activation of the Hpi moiety with trifluoroacetic acid (Tfa) leads to the formation of the thioether.[14,16] Other routes to the tryptathionine involved the reaction of tryptophan with a cysteine-sulfenyl iodide or chloride. Until now these methodologies were only used for the synthesis of phalloidin and its derivatives.[17–19]

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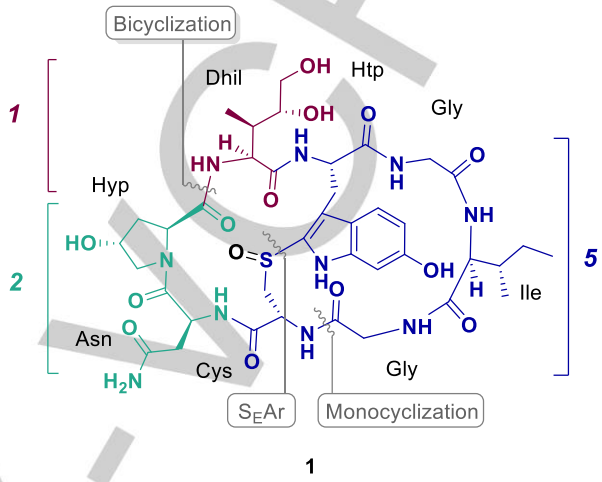

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Figure 1. Structure of α-Amanitin (**1**). The numbers in blue, turquoise and red refer to the number of amino acids of the building blocks (depicted blue, turquoise and red) employed in this total synthesis strategy [5+1+2].

The most intensively studied amatoxin is α-amanitin (**1**) (Figure 1). It contains the hydroxylated amino acids 6-hydroxytryptophan (Htp), *trans*-4-hydroxyproline (Hyp) and (3*R*,4*R*)-4,5-dihydroxyisoleucine (Dhil).[1,2] Due to its small molecular size, good solubility in aqueous buffers and its inhibition of RNA polymerase II, it received an increased attention as an antibody-drug conjugate (ADC) in cancer research in the past years.[14,20,21] In a preclinical study in 2011 conducted by the German Cancer Research Center in Heidelberg (Germany) naturally occurring α-amanitin was conjugated with the chimerized anti-EpCAM monoclonal antibody chiHEA125 to form the antibody-drug conjugate chiHEA125-Ama.[22] This therapeutic ADC targets the human epithelial adhesion molecule (EpCAM), which is overexpressed in the majority of cancer cells. The study showed that chiHEA125-Ama has a potent antitumor activity against pancreatic cancer cells. Compared with chiHEA125 two injections of chiHEA125-Ama with a dosage of 50 or 100 µg/kg (in relation to α-amanitin), showed significant tumor regression in mice.[22] Based on its promising properties for drug development there is an increasing demand for α-amanitin, which cannot be satisfied by biotechnological methods or from nature. Only last year the first total synthesis of α-amanitin has been accomplished by Perrin and coworkers, applying a sophisticated Savige-Fontana methodology as a key step for formation of the tryptathionine bridge.[23]

Herein we report an alternative and robust total synthesis of α-amanitin with the aim to be convergent and scalable in solution phase synthesis. Therefore, we applied a peptide fragment based methodology and developed straightforward syntheses to the central Dhil and Htp building blocks.

Scheme 1 shows the general retrosynthetic strategy for the total synthesis of α-amanitin (**1**) referred to as [5+1+2]-strategy.

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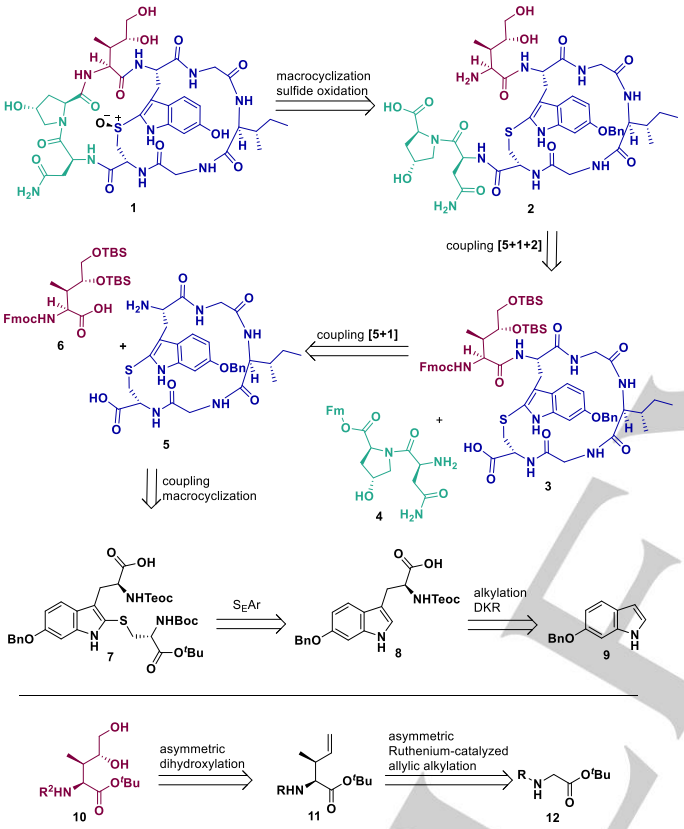
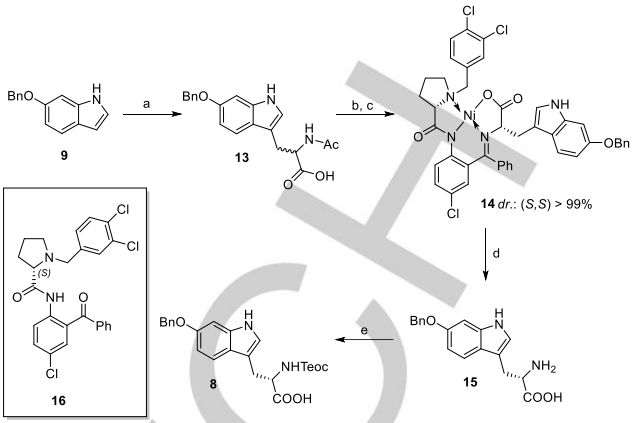
Sulfide oxidation of the tryptathionine linkage was envisioned to be introduced close to the final stages of the synthesis route, when the bicyclic structure was established by macrocyclization between Dhil and Hyp of monocyclic octapeptide **2**. This amanitin precursor should be accessible by stepwise couplings of the monocyclic building block **5** with a Dhil derivative **6** and an Asn-Hyp dipeptide **4**. It was important for reasons of scalability that both, the Dhil derivative **6** and the monocyclic tryptathionine-containing building block **5** could be synthesized on a multi-gram scale in solution phase. In our synthesis strategy, **5** is synthesized by preformation of the pseudo-orthogonally protected tryptathione linkage **7** between a 6-hydroxytryptophan (Htp) derivative (**8**) and cysteine.



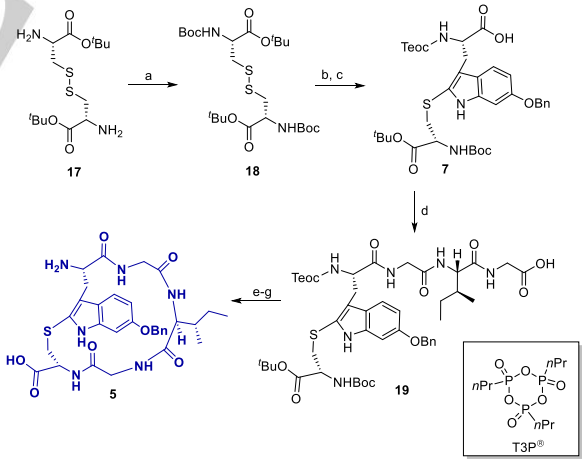
Scheme 1. Retrosynthetic analysis of α-amanitin (**1**). Final assembly of α-amanitin from three building blocks and enantioselective synthesis of the hydroxylated amino acid building blocks.

The synthesis of 6-hydroxytryptophan was not trivial and finally we decided for a dynamic kinetic resolution (DKR) route employing a chiral tridentate ligand.[24,25] Starting with 6-benzyloxyindol (**9**), alkylation using L-serine, Ac_2O and AcOH (Scheme 2) was performed, which rendered racemic tryptophan **13**.[26] After removal of the acetyl group the racemate was treated with the chiral ligand **16** and $Ni(NO_3)_2{\cdot}6H_2O$ under basic conditions, which led to nickel(II) complex **14** with a diastereomeric ratio (*dr*) of 99% (see Supporting Information). Disassembly of the nickel(II) complex **14** under acidic conditions resulted in enantiomerically pure 6-benzyloxy-L-tryptophan (**15**). Finally, the free amino acid was trapped with TeocOSu, which provided *N*-Teoc-6-benzyloxy-L-tryptophan (**8**).



Scheme 2. Synthesis of *N*-Teoc-6-benzyloxy-L-tryptophan (**8**) by dynamic kinetic resolution: a) L-serine, Ac_2O, AcOH, 75°C, 2 h, 82%; b) 40% NaOH, MeOH/H_2O, c) chiral ligand **16**, $Ni(NO_3)_2$ $6H_2O$, MeOH, reflux, 16 h, 84% over two steps, d) 6 M HCl, MeOH, 70°C, 2 h, e) TeocOSu, Et_3N, DMF, 60°C, 4 d, quant.

With 6-hydroxytryptophan derivative **8** in hand, the tryptathionine linkage was readily established by treatment of a fully protected L-cystine derivative (**18**) with sulfuryl chloride (SO_2Cl_2) (Scheme 3).[17] In the course of the reaction, cleavage of the disulfide afforded the highly reactive sulfenyl chloride monomer as an intermediate, which underwent a S_EAr reaction with Htp (**8**). We realized that protection of the *C*-terminus of **8** was not necessary for quantitative tryptathionine formation and that slow titration with the sulfenyl chloride solution prevents cysteine double substitution at Htp. Coupling of OSu-ester preactivated tryptathione **7** with a H-Gly-Ile-Gly-OH tripeptide (see Supporting Information) afforded linear pentapeptide **19**.



Scheme 3. Synthesis of the *C*- and *N*-terminally deprotected monocyclic tryptathionine peptide **5**: a) Boc_2O, $NaHCO_3$, dioxane/H_2O, r.t., 16 h, 99%; b) SO_2Cl_2, $CHCl_3$, r.t., 1 h; c) **8**, $NaHCO_3$, $CHCl_3$, 0°C to r.t., quant., d) H-Gly-Ile-Gly-OH, $CO(OSu)_2$, collidine, acetonitrile/H_2O, r.t., 2 h; e) PTSA, THF, r.t., 2 h, 70% over 2 steps; f) T3P, DIPEA, DMF, r.t., 16 h, 70%, g) TFA/H_2O (7:3), r.t., 2 h, quant.

After Boc-deprotection with *para*-toluenesulfonic acid (PTSA), macrocyclization with propanephosphonic acid anhydride (T3P) was performed. Both Teoc and *t*Bu protecting groups could be simultaneously cleaved under strong acidic conditions using TFA,

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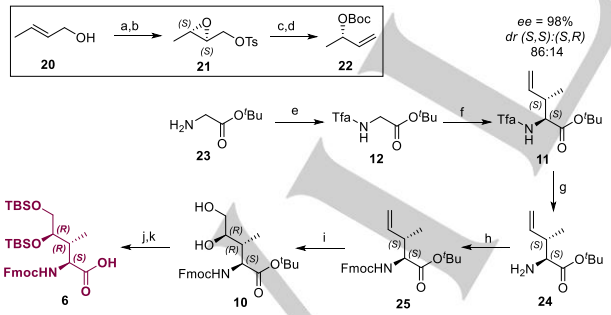
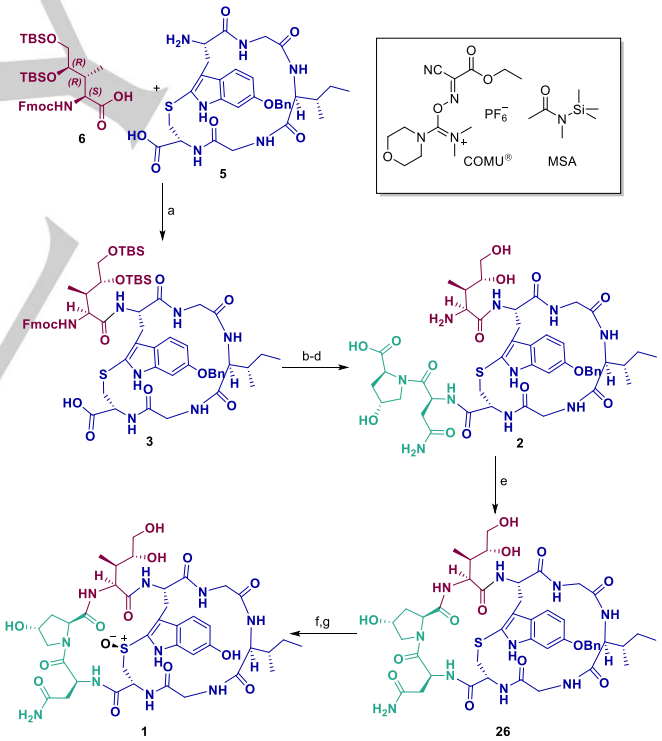
rendering monocyclic building block **5** with no decomposition of the thioether observed.

A particular synthetic challenge was the (3*R*,4*R*)-4,5-L-dihydroxyisoleucine derivative **6**, which was finally accomplished in seven steps starting from glycine *tert*-butyl ester (**23**). Quantitative trifluoroacetylation yielded Tfa-Gly-O*t*Bu (**12**) followed by ruthenium-catalyzed asymmetric allylic alkylation with **22**, in analogy to a method of Kazmaier *et al*.[27,28] While the original protocol of asymmetric alkylation employed (*S*)-3-(benzyloxy)-1-butene as alkylating agent, we used a chiral allylic carbonate (**22**), which was synthesized by Sharpless epoxidation of (*E*)-crotyl alcohol **20** followed by reductive elimination promoted by a zinc-copper-couple.[29,30] The chirality transfer promoted by allylic carbonate **22**, mainly led to the *anti*-directed formation of a fully protected 4,5-didehydroisoleucine derivative **11** with a diastereomeric ratio (*dr* = 86:14) and an enantiomeric excess (*ee* = 98%), superior to initial original reaction conditions (see Supporting Information). Then, the amino group was deprotected by reduction of the trifluoroacetyl group with sodium borohydride[31] and refurnished with the Fmoc-protecting group to give **25**. The *C*-terminal *t*Bu protecting group proved to be essential preventing lactone formation, the main threat during dihydroxylation of the fully protected didehydroisoleucine derivative **25**. The AD reaction was performed by a simple Upjohn dihydroxylation with potassium osmate and NMO. The stereoselectivity of the reaction was controllable solely by the applied solvent mixture. While dihydroxylation in a biphasic system of $H_2O/CHCl_3$ led to the formation of mainly the desired (2*S*,3*R*,4*R*)-diastereomer **10** in a 2.5:1 ratio, the undesired (2*S*,3*R*,4*S*)-diastereomer was mainly formed in a mixture of H_2O/*t*BuOH. Surprisingly, adding $(DHQD)_2PHAL$ ligand to the reaction mixture led to an impairment of the stereoselectivity towards the wrong enantiomer. Finally, the hydroxy groups of the side chain of **10** were protected with the *tert*-butyldimethylsilyl (TBS) protecting group, allowing for the mild orthogonal cleavage of *t*Bu in nearly quantitative yield in presence of an excess of TMSOTf, which resulted in the formation of Dhil building block **6** (Scheme 4).



Scheme 4. Synthesis of side chain-protected Fmoc-(3*R*,4*R*)-4,5-L-dihydroxyisoleucine (**6**) a) (+)-DIPT, $Ti(OiPr)_4$, TBHP, DCM, -20°C, 4 h; b) TsCl, Et_3N, DMAP, DCM, -10°C, 30 h, 67%; c) NaI, Zn(Cu), THF, 70°C, 2 h; d) Boc_2O, NaH, THF, 0°C to r.t., 16 h, 75%; e) ethyl trifluoroacetate, NEt_3, MeOH, r.t., 16 h, quant.; f) LHMDS, $ZnCl_2$, PPh_3, $[(p\text{-cymene})RuCl_2]_2$, **22**, THF, -72°C to r.t., 16 h, 88%; g) $NaBH_4$, MeOH, r.t., 1 h; h) FmocOSu, Et_3N, dioxane, r.t., 4 h, 82%; i) K_2OsO_4*H_2O, NMO, $CHCl_3/H_2O$, r.t., 6 h, 40%; j) TBSCl, pyridine/DMF(1:9), r.t., 24 h, 95%; k) TMSOTf, 2,6-lutidine, 0°C to r.t., 2 h, 90%.

Parallel to the synthesis of Dhil and the monocyclic tryptathionine building block a *C*-terminally 9-fluorenylmethyl ester (Fm)-protected dipeptide **4** was synthesized in solution phase (see Supporting Information) serving as the third building block for [5+1+2]-assembly. First, Dhil derivative **6** was coupled to the *N*- and *C*-terminally unprotected monocyclic pentapeptide **5** applying a protocol activating the carboxylic function of Dhil as an active ester and concomitantly increasing the nucleophilicity of the amino group of **5** by silylation with *N*-methyl-*N*-trimethylsilylacetamide (MSA) (Scheme 5).[32,33] This way no oligomerized side-products were formed. The resulting monocyclic hexapeptide **3** could then be coupled to the previously synthesized *C*-terminally Fm-protected dipeptide **4** in a one pot reaction. The final cyclization was performed after simultaneous Fmoc- and Fm-cleavage, followed by TBS deprotection with TBAF yielding monocyclic octapeptide **2**. After bicyclization using HATU to **26**, the benzyl protecting group was cleaved from the Htp side-chain of **26** applying a hard acid and soft nucleophile system.[34] Finally, the oxidation to the sulfoxide was performed using *m*CPBA according to Perrin *et al*.[23] affording the target molecule α-amanitin (**1**). The oxidation step resulted to be necessary after benzyl-deprotection and not vice versa, because of sulfoxide instability under strongly acidic conditions.



Scheme 5. Fragment couplings and cyclizations to the target molecule α-Amanitin (**1**): a) MSA, COMU, DIPEA, DMA, 0°C to r.t. 3 h; b) (a and b: onepot), H-Asn-Hyp-OFm*HCl (**4**), HATU, DIPEA, DMF, 0°C to r.t., 2 h; c) Et_2NH, DMF, 1 h, r.t.; d) 1 M TBAF, THF, r.t., 2 h, 77% over four steps; e) HATU, DIPEA, DMF, r.t., 16 h, 68%, f) BF_3*OEt_2, EtSH, r.t., 2 h; g) *m*CPBA, *i*PrOH/EtOH (2:1), r.t., 30 min, 35% over two steps.

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The final characterization of synthetic α-amanitin was performed by CD spectroscopy, which coincided well with that of the natural sample (Figure 2). The ^{1}H- and 2D-NMR-data and the co-injection on RP-C18 chromatography (see Supporting Information) of the synthetic α-amanitin were in accordance with the data of the natural one, proving undoubtedly that the α-amanitin synthesis was successful.

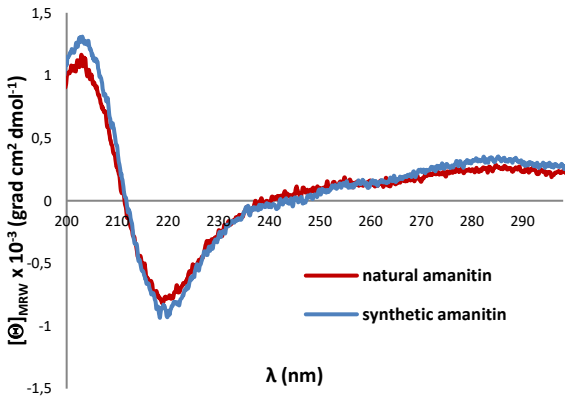

Figure 2. CD spectra of natural and synthetic α-amanitin (**1**).

In conclusion, our herein presented synthetic strategy towards α-amanitin is clearly different from the ground breaking work of Perrin *et al.*[23] in several aspects: with regard to the amino acid building blocks, we developed an independent access to 6-OH-Htp and Dhil, both non-trivial enterprises. For the latter amino acid we could rely on methodologies developed by Kazmaier *et al.* which we could engineer into a seven step synthesis with high enantiomeric excess, which so far is the shortest access to this type of amino acid. Unlike the work of Perrin *et al*., we employed a [5+1+2]-strategy under preformation of the thioether bond thus rendering a (pseudo-orthogonally) protected tryptathionine. Finally, the synthesis constitutes the first α-amanitin synthesis fully performed in solution phase. We believe that the convergent and robust synthesis, including the industrially exploitable syntheses of the amino acids Htp and Dhil, will be valuable for future therapeutic purposes in cancer therapy, when larger amounts of α-amanitin are required.

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Keywords: α-amanitin • amatoxins • amino acids • asymmetric synthesis • peptides • total synthesis • tryptathionine

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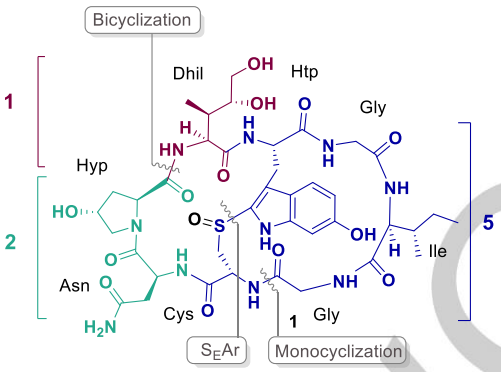
A new route to the toxic bicyclic octapeptide α-amanitin: The key steps of the convergent [5+1+2]-synthesis are the preformation of the thioether building block and the straightforward access to the enantiomerically pure non-proteinogenic amino acids 6-hydroxytryptophan and (3*R*,4*R*)-L-4,5-dihydroxyisoleucine on a multigram scale. The peptide fragment based methodology constitutes the first convergent α-amanitin synthesis fully performed in solution phase.



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