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COMMUNICATION

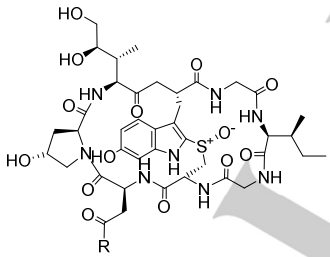
Alpha- and Beta-Amanitin Total Synthesis

Christian Lutz[a], Werner Simon[a], Susanne Werner-Simon[a], Andreas Pahl[a] and Christoph Müller*[a]

Dedicated to Prof. Heinz Faulstich for more than fifty years of amanitin research

Abstract: Alpha-Amanitin and related amatoxins are studied for more than 6 decades mostly by isolation from death cap mushrooms. The total synthesis however remained challenging due to unique structural features such as posttranslational modified isoleucine (dihydroxyisoleucine) and a tryptathion bridge dividing the macrocycle into two rings. Alpha-amanitin is a potent inhibitor of RNA polymerase II. Interrupting basic transcription processes of eukaryotes leads to apoptosis of the cell. This unique mechanism makes the toxin an ideal payload for antibody drug conjugates (ADC). Only microgram quantities of toxins, when delivered selectively to tumor sites by conjugating to antibodies are sufficient to eliminate malignant tumor cells of almost every origin. By solving the stereoselective access to dihydroxyisoleucine, a photochemical synthesis of the tryptathion precursor, solid phase peptide synthesis and macrolactamization we obtained a scalable synthetic route towards synthetic α-amanitin. This makes α-amanitin and derivatives now accessible for the development of new ADCs.

Despite of the high toxicity in humans (LD50 = 50-100 µg/kg), α-amanitin **1** remained a target structure throughout decades (**scheme 1**) [1, 2]. Recently, this compound raised a lot of interest as a potential anti-tumor agent. The toxin was first isolated as the major product of Amanita phalloides and characterized in 1978 in its beta-form **2** by crystallisation in combination with ^{1}H-NMR-studies [1, 3].

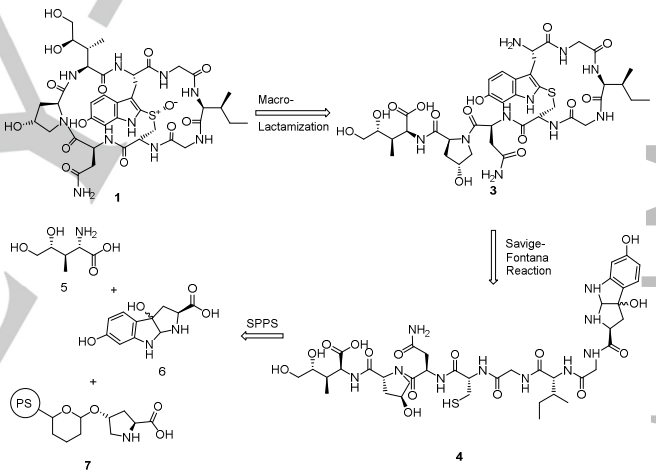


R = NH_2; alpha-Amanitin **1**
R = OH; beta-Amanitin **2**

Scheme 1. Alpha-- and beta-Amanitin.

Co-crystallisation with yeast derived RNA polymerase II (Pol II) revealed strong inhibitor properties through multiple interactions [4]. The inhibition constant Ki in HeLa cells was found to be $3x10^{-9}$ [5]. The interaction is mainly based on strong hydrogen bonds and hydrophobic interactions shown for mammalian derived Pol II with a 3000 times higher binding than to yeast Pol II [6]. Inhibition of RNA Pol II leads finally to apoptosis of the cell. In contrast to other cytotoxins α-amanitin advantageously kills both, rapidly growing and quiescent cells. This has been demonstrated in xenograft tumor models at sub-lethal doses [7] or in an antibody drug conjugate (ADC) targeted therapy of pancreatic cancer xenografts [8].

The promising, new approach towards tumor therapy increases the demand for α-amanitin to enable further studies. Since fermentation yields are low [9] and the bioprocess is not yet fully understood [10], α-amanitin gets more and more into the focus of synthetic organic chemists [2].



Scheme 2. Retrosynthetic Analysis.

Retrosynthetic analysis (**scheme 2**) and synthetic strategy considerations are mostly affected by the long history of α-amanitin chemistry [1]. Retro-macrolactamisation to **3** and most strikingly, the retro-Savige-Fontana cyclisation [11], resulted in linear octapeptide **4** as a synthetic precursor. Synthesis of the octapeptide was pre-defined by the synthetic route and protection group strategy of 4,5-dihydroxyisoleucine (DHIL) **5**. This pretended a C-terminal coupling to immobilized hydroxy-L-proline **7** at the beginning of the SPPS as previously described in the phalloidin synthesis [12]. The remaining five amino acids were assembled by the more favourable Fmoc strategy plus 3a,6-dihydroxy-hexahydro-pyrrolo[2,3-b]indole-2-carboxylic acid (Hpi) **6** finally. Key building blocks are DHIL **5** and Hpi **6** therefore.

DHIL **5** carries three adjacent stereocenters to be assembled in the right configuration. A first racemic synthesis goes back to 1957 [13]. A major hurdle of the DHIL synthesis is the possible

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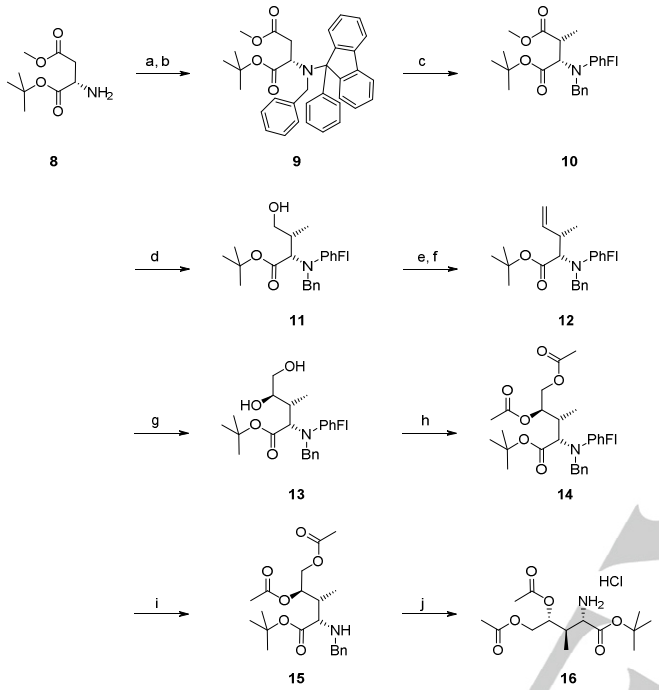
spontaneous lactonization at any stage, making the right choice of protecting groups crucial.

Newer approaches e.g. applied the Mannich reaction [14, 15, 16], Claisen rearrangement [2b, 17] or a Brown crotylation in combination with the Strecker amino acid synthesis [2b]. The common diol motif was mostly introduced by well known dihydroxylation procedures [18]. These DHIL approaches resulted in the synthesis of α-amanitin and γ-amanitin so far [2a, 2b].



Scheme 3. Dihydroxyisoleucine (DHIL) Synthesis. Conditions: a) PhCHO, $NaBH(OAc)_3$, THF, 0°C; b) PhFlBr, $Pb(NO_3)_2$, H_3CCN, RT, 57%, 2 steps; c) LiHMDS, MeI, THF, -30°C; d) $LiAlH_4$, THF, 0°C to RT, 60% 2 steps; e) $(COCl)_2$, DMSO, -80°C, then Et_3N; f) $Ph_3PCH_3^+B^r$-, NaH, DMSO, RT, 83%, 2 steps; g) AD-mix beta H_2O/^{t}BuOH, 35°C, chrom. Purification, 68% major isomer, 96.8%ee; h) Ac_2O, Et_3N, CH_2Cl_2, 82.5%; i) F_3CCO_2H in CH_2Cl_2, 86%; j) H_2/Pd/C, 1N HCl in EtOH, 92.8% (Abbr.: PhFlBr; Phenylfluorenylbromid)

We choose to start with 4-methyl aspartate ester as building block, readily available from commercial source. Full protection was accomplished by tert-butyl esterification at C-1 under standard conditions **8**[19] (**scheme 3**), reductive amination with benzaldehyde and phenyl fluorenyl protection of the amine **9** [20]. Regio- and stereoselective methylation with LHMDS/MeI at C-3 gave in a 5:1 selectivity the desired isomer **10** while the combination of KHMDS/MeI favors the opposite due to chelate formation of the intermediate potassium salt as described elsewhere [21]. This worked out for tert-butyl esters as well, facilitating regioselective reduction of the methyl ester with $LiAlH_4$ to alcohol **11** in the following step.

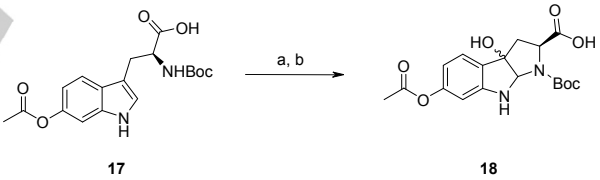
The low alkylation selectivity was ruled out by $LiAlH_4$ reduction to alcohol **11** (Scheme 4). While the undesired diastereomer leads to a facile lactonization product, the desired isomer halts at the alcohol stage **11** and can be separated easily in high diastereomeric purity.

Olefin homologation under standard conditions by Swern oxidation and Wittig reaction to **12**, was followed by AD-mix beta dihydroxylation with a preference (70:30) for the desired isomer **13**. Isomers were easily separated by chromatographic resolution. After acetylation **14**, the phenyl fluorenyl group was deprotected under acidic conditions to **15** prior benzyl group removal by hydrogenation to yield amino acid **16**. A twostep procedure became necessary due to removal of the phenyl fluorenyl protecting group from the product. Lactonization was not observed throughout the synthesis.

Besides the DHIL synthesis, the tryptathione bridge dissecting the macrocycle into two rings is quite challenging. Introduction of a pre-build tryptathion starting from L-Trp and L-Cys is possible, but quite tedious. Synthesis needs sulfenyl chloride or iodine pre-activated Cysteine-dimer reacting with Tryptophan [22]. An orthogonal protection group strategy becomes then necessary to build up the framework.

Closing the ring after assembling 8 amino acids is more straightforward. Besides exotic mercury [23] and successful iodine cyclisation [24] in the synthesis of phalloidin structures, amatoxin synthesis is strongly related to a reaction first described by Savige and Fontana [25] and applied by Zanotti [26]. Key intermediate is the oxidized form of L-Tryptophan derivative Hpi, that reacts under acidic conditions prompt and neatly with nucleophiles such as sulfur from L-cysteine to form the right-hand ring of α-amanitin. Hpi is accessible by peracid or DMDO oxidation [27],[28] and was applied in the first total synthesis of α-amanitin by Perrin et al [2b]. Unfortunately, these methods use hazardous oxidizers and therefore are difficult to use on a larger scale.

A further access has been shown by photooxygenation of L-tryptophan with oxygen [29]. A very mild access with high functional group tolerance.



Scheme 4. 6-Acetoxy 3a-hydroxy pyrrolo indene (Hpi) Synthesis. a) hν/sens/O_2; b) Me_2S, 69%

Thus, we were able to oxidize 6-acetoxy-N-Boc-L-tryptophan (**17**) directly to a mixture of cis-/trans- 6-acetoxy-N-Boc-hydroxy-pyrroloindene (**18**, **scheme 4)**. The cis-/trans- mixture can be used without any implication of the stereochemical outcome of the cyclisation/tryptathion formation [30]. However, 6-hydroxy-L-tryptophan is of limited commercial availability which makes alternative routes attractive (**scheme 5**).

Thus, easily accessible 6-benzyloxy indene-3-carbaldehyd (**19)** was protected with Cbz and reacted with a Horner-Wadsworth-Emmons reagent to yield olefin **20** with an E/Z ratio of 98:2. After enantioselective hydrogenation tryptophan derivative **21** was obtained in 95.6 %ee [31]. After hydrogenative deprotection to **22** and acetylation of the 6'-HO-position, precursor **17** was obtained.

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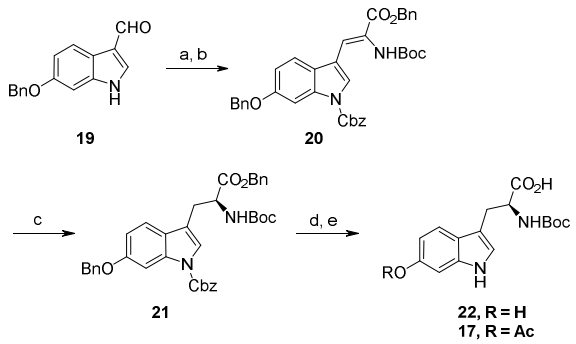
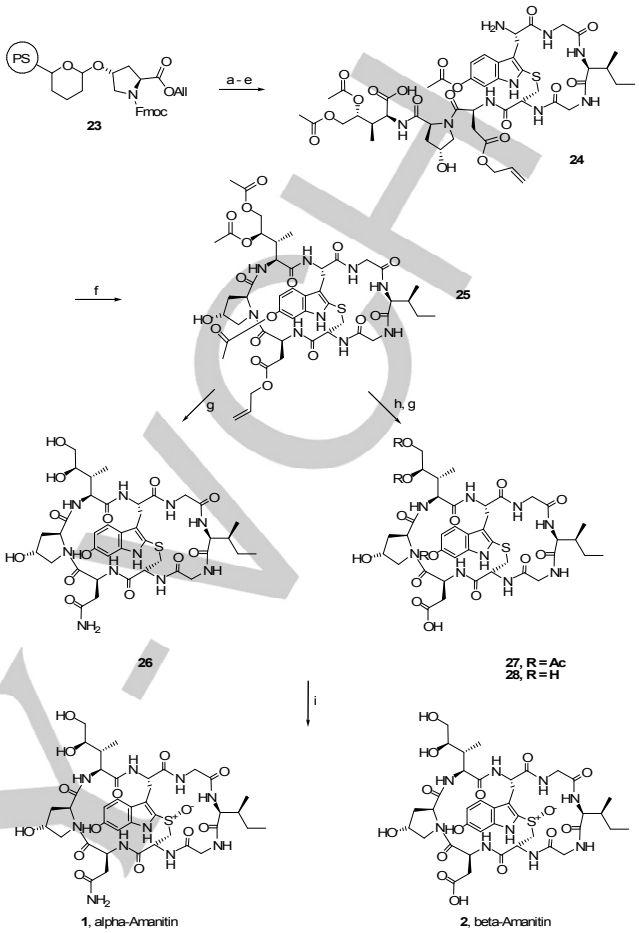


Scheme 5. 6-Hydroxytryptophan Synthesis. Conditions: a) CbzCl, Et_3N, DMAP, RT, 76%; b). Boc-2-Phosphonoglycine benzyl dimethyl ester, DBU, 73%; c) [Rh((*R*,*R*)-DiPAMP)COD]BF_4; H_2 d) Pd/C, H_2; e) Ac_2O,1N NaOH, 68%, 2 steps

Having the key building blocks in hand, SPPS (**scheme 6**) was straightforward. FmocHypOAll was immobilized on THP resin **23** (1.0 mmol/g) [22]. After allyl deprotection of the resin, DHIL was coupled C-terminal by PyBOP/DIEA activation under microwave conditions [50W, 10 min] and N_2 bubbling. After Fmoc deprotection with piperidene, the following amino acids, Fmoc-Asp(OAll)OH, FmocCys(Trt)OH, FmocGlyOH, FmocIleOH, FmocGlyOH were coupled iteratively, followed by 6'-Acetoxy-N-Boc-Hpi **18** as final building block. The octapeptide is cleaved off the resin with TFA for 15 minutes, washed into a reaction flask and stirred until complete cyclisation of the first ring (Savige-Fontana reaction, 30 minutes) to yield intermediate **24**. The bicycle **25** is formed by macrocyclization with DPPA/DIEA under dilute condition. The acetyl and allyl protection groups were cleaved off at once with ammonia in methanol to yield **26**. In case of β-amanitin, the allyl ester is selectively removed with $Pd(PPh_3)_4/HCO_2H$/TEA to **27**, followed by deprotection of the three acetyl groups with ammonia to obtain **28**. Intermediates were purified by preparative HPLC (some purification steps can be omitted or substituted by precipitation due to low side product formation). Final sulfoxidation was performed with mCPBA yielding the desired *R*-**1** or *R*-**2** over the *S*-diastereomer in a ratio of 2:1[2b]. Both isomers can be easily separated by preparative RP-HPLC. The obtained α- and β-amanitin **1**, **2** show identical NMR and CD spectra compared to their natural counterparts (see supplementary material).

In conclusion, we presented a facile route to natural α-and β-amanitin (**1** and **2**). Key building blocks, such as DHIL **16** and Hpi **22** are readily available starting form natural building blocks. The two additional stereocenters of DHIL **16** are accessible by selective alkylation and oxidation procedures on a multi-gram scale. Assembling of the octapeptide precursor was performed by microwave assisted coupling but can be easily scaled up to traditional shake flask methodology. The final steps (Savige-Fontana cyclisation, macrolactamisation and acetyl deprotection) ran smoothly without significant formation of side products. Selectivity of the sulfoxidation needs further improvement, however, recycling of the undesired *S*-sulfoxide is possible by reduction to 30 and 32 [32]. Today, the process is already up-scaled and able to deliver Amatoxin derivatives on a multi gram scale in GMP quality.



Scheme 6. α- and β-Amanitin Synthesis. Conditions: a) $Pd(PPh_3)_4$, DMBA, DCM, RT, o/n; b). **16**, PyBOP, *i*Pr_2NEt, HOBt, DCM/DMF 1:1, Microwave; c) 20% Piperidine, NMP, Microwave; d) steps b, c iteratively repeated with FmocAsp(OAll)OH, FmocCys(Trt)OH, FmocGlyOH, FmocIleOH, FmocGlyOH, **22**; e) TFA neat, 30 minutes, 47%; f) DPPA, *i*Pr_2NEt, dilution, 48%; g) 7N NH_3 in MeOH, (**30**, 73%; **31**, 57%); h) $Pd(PPh_3)_4$, HCO_2H/TEA, RT, 30 min, 67%; i) mCPBA (**1**, 54%; **2**, 47%)

This can be seen as a cornerstone for the commercial use of amatoxin based antibody drug conjugates (ADCs), so called antibody targeted amatoxin conjugates (ATACs). After attachment of stable or cleavable linkers at different functional groups of α-amanitin, toxin-linker derivatives are conjugated to antibodies [33] or small molecular targeting formats. ATACs show very promising results as highly potent and selective anti tumor agents [8].

Experimental Section

Detailed methodology is provided in the supporting information with accompanying spectra and other key data reports.

Keywords: α-Amanitin • β-Amanitin • amino acid • peptides • SAvige-Fontana reaction • solid phase synthesis • total synthesis

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COMMUNICATION

A scalable route to α- and β-Amanitin: The bicyclic octapeptide is build up by solid phase assembling of a linear octamer followed by two consecutive cyclisation steps in solution. The key non-proteinogenic amino acids, 6-hydroxy tryptophan and (3R, 4R)-dihydroxyisoleucine are accessible via multi step synthesis on a multi-gram scale. Accessibility of Amatoxins is a cornerstone for the commercial development of new payload linkers for antibody drug conjugates.

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