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FULL PAPER

Design, Synthesis, and Biochemical Evaluation of Alpha-amanitin Derivatives Containing Analogs of the *Trans*-hydroxyproline Residue for Potential Use in Antibody-drug Conjugates

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Supporting information for this article is given via a link at the end of the document.

Abstract: Alpha-amanitin, an extremely toxic bicyclic octapeptide extracted from the death-cap mushroom, *Amanita phalloides,* is a highly selective allosteric inhibitor of RNA polymerase II. Following on growing interest in using this toxin as a payload in antibody-drug conjugates, herein we report the synthesis and biochemical evaluation of several new derivatives of this toxin to probe the role of the *trans*-hydroxyproline (Hyp), which is known to be critical for toxicity. This structure activity relationship (SAR) study represents the first of its kind to use various Hyp-analogs to alter the conformational and H-bonding properties of Hyp in amanitin.

Introduction

Alpha-amanitin, isolated over 70 years ago, is the principal toxin in *Amanita phalloides* - the notorious "death-cap" mushroom. With a rich scientific history,[1] it is one of the deadliest toxins found in nature (LD_{50} = 50-100 μg/kg in humans). Its unique bicyclic structure comprises a 6'-hydroxytryptathionine-(*R*)-sulfoxide staple along with two oxidized amino acids that are considered critical for cytotoxicity: *trans*-4-hydroxyproline (Hyp) and (2*S*,3*R*,4*R*)-4,5-dihydroxyisoleucine (DHIle).[2] Featured in most modern biochemistry textbooks, α-amanitin is a highly selective allosteric inhibitor of eukaryotic RNA polymerase II (Pol II) with a K_i value of 1-10 nM,[3] the inhibition of which leads to rapid proteolytic degradation of Pol II and cell death.[4]

Because Pol II-catalyzed transcription is essential for cellular function, α-amanitin kills dividing *and* quiescent cells by a unique mechanism of action. It is precisely this generalized cytotoxicity that makes α-amanitin an attractive payload for antibody drug conjugates (ADCs).[5] Following an early report on an amanitin-antibody conjugate against Thy 1.2 antigen towards T lymphoma S49.1 cells,[6] Moldenhauer *et al.* demonstrated the extraordinary promise of α-amanitin as a payload for ADC development; an anti-EpCAM antibody-amanitin conjugate remarkably cured 60% (3 of 5) mice with pancreatic tumor xenografts,[7] paving the way for HDP-101, an amanitin-based ADC for the treatment of multiple myeloma and the first amanitin-based ADC that is advancing towards clinical trials.[8] Others have explored targeted amanitin conjugates underscoring interest in amanitin and its chemistry.[9]

With this interest in using α-amanitin for therapeutic applications, new knowledge as to the molecular basis of toxicity will be essential for designing new toxicophores for therapeutic applications. XRD studies on α-amanitin-Pol II co-crystal obtained by Kornberg and coworkers have suggested multiple interactions between this toxin and the bridge helix of Pol II from S. cerevisiae (**Figure 1**, A)[10] including numerous interactions with backbone amides, π-stacking/π-cation interactions with the hydroxy-tryptathionine, and putative H-bonds to the hydroxyl groups of Hyp and DHIle. These results were corroborated by cryo-EM structures obtained by Cramer and coworkers (**Figure 1**, B).[11]

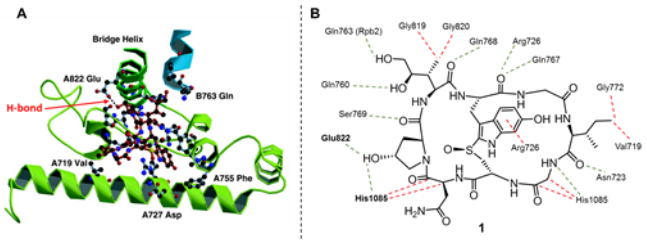

Figure 1. (**A**) Co-crystal structure of α-amanitin bound to yeast (*S. cerevisiae*) Pol II at 2.8 Å resolution (reproduced from Bushnell *et al*. ref. 10 with permission from Proc. Natl. Acad. Sci.). The H-bond between Glu822 of Pol II and the hydroxyl group of Hyp is shown. (**B**) Observed interactions between different residues of α-amanitin **1** and the backbone of Pol II (Brueckner *et al.*).[11] Hydrogen bonds are shown in dashed green lines and other interactions are marked with dashed red lines. Note the H-bonding interactions between the hydroxyl group of Hyp and Glu822 and His1085 of the Rpb1 subunit of Pol II.

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Recent cryo-EM structures on porcine (*S. scrofa*) RNA Pol II show that Glu845 plays the analogous role of Glu 822. Yet an H-bond acceptor, analogous to His1085, is not evident this addition interaction is not clearly supported in the known structures of mammalian RNA Pol II.[12]

These fascinating structural studies cohere in part with earlier structure activity relationships (SARs) reported in the 1980s, based on naturally-sourced amatoxins. For example, deoxygenation of the (*R*)-sulfoxide to the thioether or oxidation to the sulfone resulted in no loss of activity in the case of the 6'-*O*-methyl ether of α-amanitin.[13] Naturally occurring amanullin and proamanullin, which lack the hydroxyl groups on DHIle or on both DHIle and Hyp respectively, show K_i values of 10-40 nM (near-native) and 5-20 μM (greatly attenuated) respectively. [2a, 14] The synthetically accessible "tetradeoxy-amanitin" (lacking the sulfoxide, the 6'-hydroxy group on the tryptathionine, and where Ile replaces DHIle) was reported to have a K_i value of 80 nM in an *in vitro* transcription assay with calf-thymus polymerase,[14a] but a much elevated K_i of 1 μM in a different report on drosophila RNA Pol II [2b] (despite considerable interspecies homology *cf. B. taurus*, *S. scrofa, C. capitata*). Yet these values had been obtained using transcription assays that often differ in terms of the species of Pol II used in the assay as well as how the assays were conducted. Nevertheless, taken together, these studies, along with crystal/cryo-EM studies suggest that the Hyp is a major contributor to toxicity.

In Lipscomb's structure,[15] the *trans*-(4*R*)-hydroxyproline exists in the *trans*-amide bond and adopts the classic C_γ-*exo* ring pucker conformation commonly seen for Hyp in collagen. The C_γ-*exo* ring pucker is favored by *trans*-oriented electron-withdrawing groups (EWGs) at the 4-position, that intensify the gauche interaction between the amide bond and the EWG (**Figure 2**).[16]

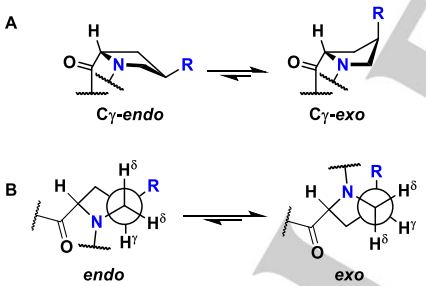

Figure 2. (**A**) *Exo* and *endo* ring puckers observed in the proline residue of a *trans*-4-substituted proline-containing peptide; (**B**) hyperconjugation of the σ(C-H^δ) orbital and the electron-deficient σ*(C-R) where (R = EWG).

As such, the hydroxyl group may contribute to amanitin toxicity, not only *via* H-bonds but *via* a C_γ-*exo* conformation that may reinforce subtle conformational differences throughout the entire macrocycle. Nevertheless, the precise role of this hydroxyl group, either in terms of H-bonding or ring-puckering remains entirely unaddressed in the vast literature on amanitin derivatives. Furthermore, a systematic SAR investigation based on rationally designed Hyp analogs represents a worthy challenge that has yet to be undertaken, likely owing to synthetic inaccessibility of DHIle.

In 2018, we completed the first total synthesis of α-amanitin providing an enantioselective synthesis of (2*S*,3*R*,4*R*)-4,5-dihydroxyisoleucine on moderate scale.[17] In 2020, two additional total syntheses highlighted the need for synthetic access.[18] With the increased interest in α-amanitin, here we begin to tackle SAR profiling of the Hyp with choice analogs introduced into new toxin analogs based on structural and conformational considerations.

Results and Discussion

We based our analysis on the synthetically more accessible "dideoxy-amanitin" (6'-deoxy-S-deoxo-α-amanitin) (**2**) lacking both the 6'-OH and the (*R*)-sulfoxide, where the thioether is known to be equipotent in cytotoxicity assays,[13, 19] confirmed by us and others.[9d, 17, 20] Typically, toxins constructed with a simple tryptathionine staple show near-native potencies.[2a, 14a, 21] Hence, we prepared the dideoxy-amanitin scaffold so as to make this comparison internally consistent across all proline analogs studied here.

In choosing 4-substituted prolines, we considered studies on collagen that employed conformationally biased (4*R*)-substituted prolines that have questioned the contribution of the H-bonding by the Hyp to embedded water molecules or to carbonyls on opposing collagen strands.[22] Indeed, (4*R*)-substituted prolines that are incapable of H-bonding, e.g. 4-fluoro-, 4-chloro-, 4-azido-proline form highly stable collagen helices[23] as reviewed,[24] suggesting that ring-pucker contributions are more important than H-bonding for determining the structural integrity of collagen. This bias challenges analog development in amanitin because of the need to also address defined H-bonds to Glu845 with a suitable functional group, while also ensuring a C_γ-*exo* conformation that is seen in the crystal structures of α- and β-amanitin.[14a, 15c, 19]

To maintain the conformational bias of Hyp while affording H-bonding functionalities, we looked at comprehensive work by Zondlo *et al*.[25] and synthesized five *trans*-4-substituted prolines for insertion into amanitin. Hence, we considered: (*i*) potential for H-bond donation or acceptance and (*ii*) the ring pucker induced by the presence of a *trans*-4-substitutent on the proline residue. Given limitations in throughput, the cyano-, amino-, mercapto-, and guanidino- analogs appeared compelling due to their potential to form at least one H-bond with Glu845. It was further anticipated that the guanidine would form bifurcated H-bonds or alternatively a salt-link with Glu845.[26] The use of the amino-proline was rationalized similarly in terms of favorable electrostatic interactions and/or H-bonding interactions. Additionally, we investigated two protected intermediates, Acm-protected *trans*-4-mercapto-proline and Boc-protected *trans*-4-guanidino-proline along with a *trans*-4-methylcarbamoly-proline, that adventitiously formed upon azide reduction (*vide infra*), all of which provide H-bonding functionalities (**Table 1**).

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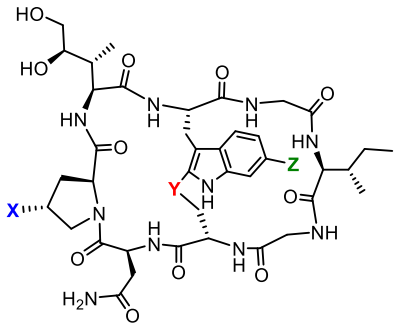
Table 1. Proposed analogs of Hyp for incorporation into amanitin. Compound 1 represents the natural product, α-amanitin, while 2 shows dideoxy-α-amanitin, which is the basis for our synthetic analogs. Compound 32 is an adventitious by-product that was not initially proposed as an analog.



Cmpd.	X	Y	Z
1	OH	(*R*)-SO	OH
2	OH	S	H
3	N_3	S	H
4	CN	S	H
5	SH	S	H
6	NH_2	S	H
7	Gdn	S	H
8	SAcm	S	H
9	Boc-Gdn	S	H
32	MeOCONH	S	H

In terms of ring-puckering, whereas the mercapto-proline prefers a C_γ-*endo* conformation in approximately 5:1 ratio over the C_γ-*exo*, [27] both the amino- and guanidino-prolines (extant at physiological pH as ammonium and guanidinium cations) are known to strongly favor a C_γ-*exo* conformation.[28] *Trans*-4-amido prolines as well as carbamoylated ones (*N*-Boc-modified) are also known to adopt a C_γ-*exo* conformation,[29] leading us to rationalize testing a methylcarbamate **32** that formed serendipitously upon azide reduction (*vide infra*). The cyano-proline, which is strongly electron withdrawing, is expected to favor the C_γ-*exo* conformation, and has the potential to accept an H-bond, albeit weakly.[30] Finally, the azido-proline, also known to favor the C_γ-*exo* conformation,[31] provides a control for conformation in the absence of H-bonding. The logical application of 4-fluoroproline was not carried forward in this analysis as it did not provide a cytotoxic analog of dideoxy-amanitin, and in our hands proved difficult to synthesize (data not shown).

The synthesis of the Hyp analogs began with preparation of *cis*-*N*$^\alpha$-Boc-4-hydroxyproline methyl ester (*cis*-Boc-Hyp-OMe, **12**) from the commercially available *trans*-*N*$^\alpha$-Boc-4-hydroxyproline (Boc-Hyp, **10**); Boc-Hyp was subjected to an intramolecular Mitsunobu reaction using PPh_3 and DIAD to afford the lactone of *cis*-Hyp **11**.[25] An azide-assisted saponification of this lactone with methanol afforded the methyl ester of *cis*-Boc-Hyp **12**.[32] Subsequently, the hydroxyl group was mesylated and the resulting compound was subjected to S_N2 conditions to yield the desired *trans*-isomer of various analogs (**Figure 3**).

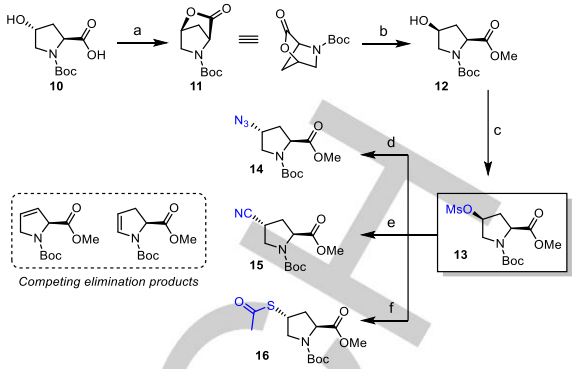


Figure 3. Synthesis of Boc-protected methyl esters of azido-, cyano- and mercapto-proline analogs. Reagents and conditions: a) PPh_3 (1.2 eq.), DIAD (1.2 eq.), THF, 0°C to RT, 20 h, 85%. b) NaN_3 (2.0 eq.), MeOH, 40°C, 16 h, 75%. c) MsCl (1.6 eq.), Et_3N (1.4 eq.), DCM, 0°C, 16 h, 95%. d) NaN_3 (2.0 eq.), DMSO, 80°C, 4 h, 79%. e) KCN (1.5 eq.), DMSO, 80°C, 4 h, 30%. f) KSAc (1.3 eq.), DMF, 70°C, 4 h, 52%.

For the synthesis of azido- and cyano-proline analogs, their corresponding methyl esters were saponified to the free acid, and the Boc protecting group was swapped with Fmoc to yield SPPS compatible monomers **21** and **22** (**Figure 4**, A). In the case of mercapto-proline, the thioacetate group and the methyl ester of **16** were concomitantly saponified, the Boc protecting group was removed, then an Acm (acetamidomethyl) protecting group was introduced on the free thiol. Finally, the free amine was protected with Fmoc to yield the fully protected monomer **26** (**Figure 4**, B).

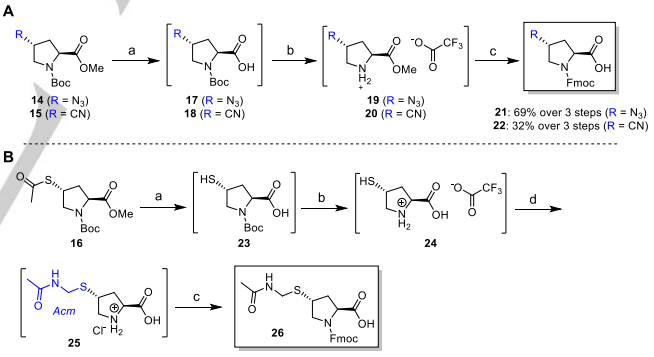


Figure 4. Synthesis of fully protected, SPPS compatible monomers of: (**A**) azido-, cyano-proline and (**B**) mercapto-proline. Reagents and conditions: a) LiOH (20 eq.), THF/H_2O (1:1), 0°C, 16 h. b) TFA/DCM (1:2), RT, 30 min. c) Fmoc-OSu (1.1 eq.), $NaHCO_3$ (2.0 eq.), 1,4-dioxane/H_2O (2.3:1), RT, 3 h. d) (*N*-hydroxymethyl)acetamide (1.2 eq.), 12 M aq. HCl (1.0 eq.), H_2O, 0°C to RT, 48 h, 25% over 4 steps.

Following a solid phase strategy similar to our reported total synthesis, we incorporated the three aforementioned monomers into the corresponding dideoxy-amanitins (**Figure 5**). To summarize, Hyp along with the synthetic Hyp analogs were separately loaded on 2-chlorotrityl chloride (CTC) resin, followed by the coupling of Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Gly-OH and Boc-Fpi-OH (**30**), where Fpi = 3a-fluoro-hexahydropyrrolo-[2,3-*b*]indoline,[33] to afford the linear heptapeptide. Treating the resin with TFA/DCM (1:1) resulted in the global deprotection of the acid-labile protecting groups and tryptathionine formation *via* the Savige-

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Fontana reaction[34] to yield monocyclic heptapeptides of various amanitin analogs (**27c**, **28c**, **29c**). Next, the DHIle **31** was grafted onto the *N*-terminus, followed by *in situ* removal of Fmoc and TBS protecting groups. The resulting monocyclic octapeptides (**27d**, **28d**, **29d**) were macrolactamized to yield the final bicyclic octapeptides containing different Hyp analogs (**3**, **4** and **8**).

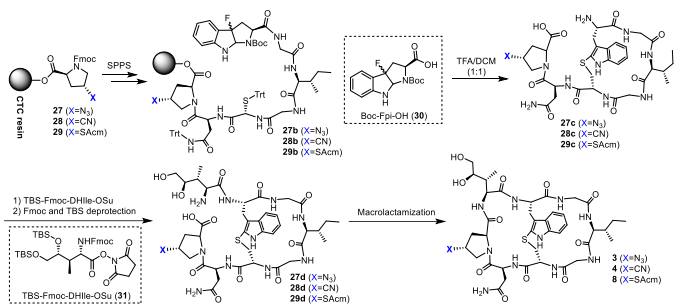

Figure 5. Synthesis of amanitin derivatives containing three Hyp analogs: azido-, cyano- and SAcm-prolines. A solid-phase peptide synthesis involving an Fmoc strategy was utilized (see Supporting Information for reagents and reaction conditions).

To synthesize analog **5** (mercapto-proline), the Acm-protected intermediate **8** was treated with a large excess of $PdCl_2$ in 6M aq. guanidinium hydrochloride (Gdn·HCl) followed by quenching with DTT to remove the Pd (**Figure 6**, A).[35] To prepare the amino-proline analog from the azido-prolyl amanitin, Staudinger reduction conditions were initially employed using PPh_3 in aqueous DMSO.[36] To our surprise, instead of obtaining the desired amino analog, we obtained a byproduct that we characterized to be the methyl carbamate of the amino-proline residue (**32**), based on the mass spectrometry and preliminary ^{1}H-NMR studies showing a characteristic singlet (see Supporting Information). There is literature precedent for the reaction of organic azides with triphenylphosphine and CO_2 to give isocyanates that may in turn react with nucleophilic solvents (i.e. MeOH in this case, see Supporting Information for mechanism).[37] Without prejudice, we incorporated this derivative into our panel of analogs for biochemical characterization. Attempting alternative conditions for the reduction, the azide group on **3** was successfully reduced to an amine **6** using DTT in DMSO (**Figure 6**, B).[38]

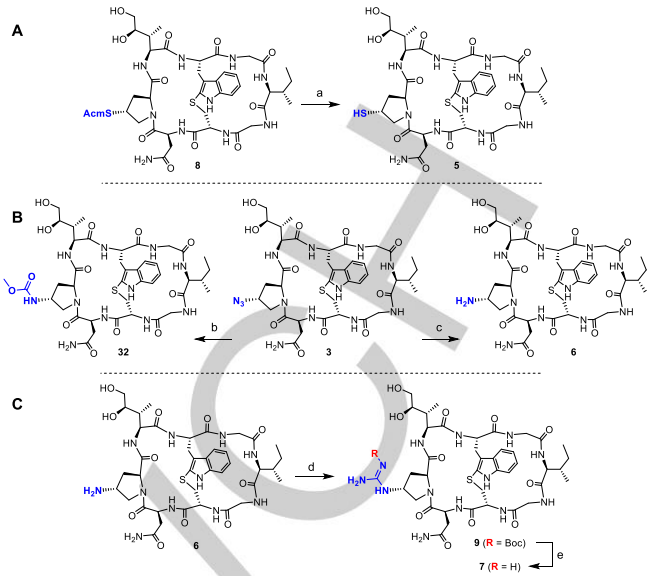

Figure 6. (**A**) Removal of Acm to afford the mercapto-proline analog **5**. (**B**) Reduction of the azide under Staudinger conditions (PPh_3, DMSO) to yield the speculated methyl carbamate analog **32**, and using DTT to obtain the amino-proline analog **6**. (**C**) Guanidinylation of amino-proline-amanitin **6** to afford the Boc-protected (**9**) and unprotected guanidino-proline **7** amanitins. Reagents and conditions: a) $PdCl_2$ (20 eq.), 6 M aq. Gdn.HCl, 37°C, 30 min. b) PPh_3 (12 eq.), DMSO, RT, 16 h. c) DTT (5.0 eq.), DMSO, RT, 3 h. d) i. *N*-Boc-*N*'-TFA-pyrazole-1-carboxamidine (6.0 eq.), DIPEA (2.5 eq.), THF/DMSO (2:1), RT, 16 h. ii. K_2CO_3 (13 eq.), MeOH/H_2O (2.5:1), RT, 3 h. e) TFA/DCM (1:2), RT, 1 h.

To prepare the guanidino analog **7**, amanitin **6** was reacted with *N*-Boc-*N*'-TFA-pyrazole-1-carboxamidine in THF/DMSO in the presence of DIPEA.[39] The resulting doubly protected guanidine was treated with K_2CO_3 in MeOH and H_2O to remove the TFA protecting group, affording intermediate **9**. Finally, the Boc protecting group was removed using TFA in DCM (1:1) to yield an amanitin analog **7**, containing a guanidino-proline residue (**Figure 6**, C). Lastly, to prepare the dideoxy-amanitin (**2**), we simply followed our previous reports on the synthesis of amanitin starting with Hyp(O*t*Bu) (see Supporting Information).

Eight analogs were purified and compared to both α-amanitin (**1**) and the more chemically similar dideoxy-amanitin (**2**). To compare the overall conformation of these amanitins, circular dichroism (CD) spectra of all the analogs were obtained in two solvents: 1) MeOH, which is the standard solvent used to evaluate amatoxins,[2] and 2) MeOH/0.1% aqueous formic acid (10:1) at pH 4.5. Although **2** showed a slightly different spectrum from the others (in MeOH), all analogs exhibited similar CD spectra (**Figure 7**, A), with a negative Cotton effect generally observed between 215 and 250 nm, and a positive Cotton effect above 250 nm.

Curiously, in the acidic environment of MeOH/H_2O, pH 4.5, four analogs (N_3-, CN-, SH- and Acm-S-proline amanitins: **3**, **4**, **5**, and **8** respectively) demonstrated similar CD spectra, while the others (amino-, Gdn-, Boc-Gdn-, and methylcarbamoyl-prolines: **6**, **7**, **9**, and **32** respectively) showed highly disparate spectral signatures (**Figure 7**, B). Interestingly, unlike the other analogs, **2** seemed to vary minimally upon this solvent change.

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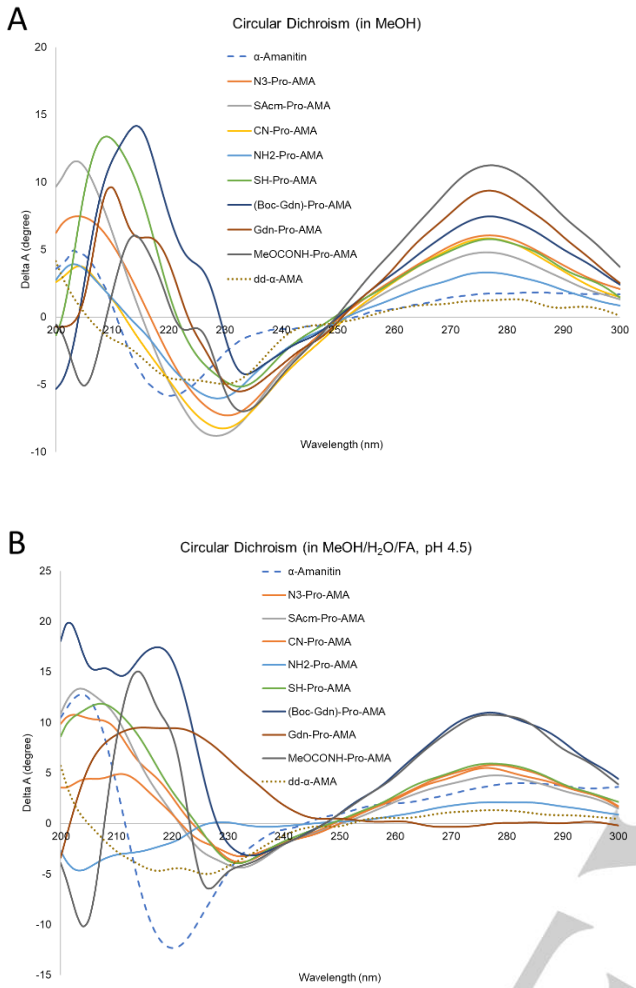

Figure 7. Circular dichroism (CD) spectra of α-amanitin and the synthetic amanitins containing analogs of Hyp. (**A**) CD in MeOH. (**B**) CD in MeOH/(0.1% FA in H_2O), 10:1 at pH 4.5.

We then investigated cytotoxicity using CHO (Chinese hamster ovary) cells as they are readily killed by α-amanitin (**1**, K_i ca. 0.5 μM) even though they do not overexpress the organic anion-transporting protein (OATP) implicated in active toxin transport.[40] To assess the cytotoxicity of these synthetic analogs, CHO cells were treated with various concentrations ranging from 0.078 to 20 μM, and the percentages of viable cells were measured using an MTT colorimetric assay (see Supporting Information). To our disappointment, none of the synthetic analogs exhibited any appreciable level of toxicity towards CHO cells, even at concentrations as high as 20 μM (**Figure 8**, A and B). As controls, IC_{50} values of 0.3 μM that were obtained for both α-amanitin and dideoxy-amanitin in this assay were in line with the values previously reported by us[9d, 17] and others[2a, 14] (**Figure 8,** C).

In a separate experiment, the unprotected analogs (**3**-**7** and **32**) were tested against human embryonic kidney 293 (HEK293) cells, with and without overexpression of the OATP1B3 protein, to investigate the effect of this anion-transporting protein on the cytotoxicity of the synthetic analogs. While some analogs showed slightly elevated toxicity against the HEK293 cells, which overexpress OATP1B3, this increase was marginal, and no conclusive IC_{50} values could be obtained for these analogs (**Figures ESI.7** and **ESI.8** in Supporting Information).

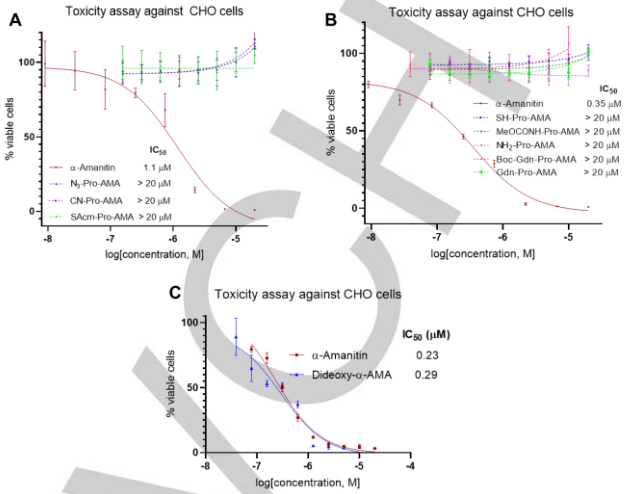

Figure 8. MTT colorimetric assay for evaluation of the toxicity of the synthetic analogs against CHO cells. (**A**) azido, Acm-S- and cyano-proline analogs, (**B**) mercapto, MeOCONH, amino, Boc-Gdn and Gdn-proline analogs; (**C**) controls – dideoxy-amanitin and α-amanitin.

Surprised by these findings, we questioned whether poor internalization of certain derivatives, in particular **6** (amino-proline) and **7** (guanidino-proline), resulted in an apparent lack of toxicity. To address this hypothesis, all synthetic analogs (with the exception of the putative MeOCONH-proline analog **32** due to limited amounts) were then subjected to an *in vitro* transcription assay using HeLaScribe® nuclear extract containing Pol II and all other transcription factors using a DNA template containing a strong cytomegalovirus (CMV) promoter (see Supporting Information for detailed procedures).[41]

To test Pol II inhibition, increasing concentrations of α-amanitin (**1**, 1 to 100 nM) were added to the transcription reaction. Following autoradiography, an inhibition curve for α-amanitin was obtained, with an IC_{50} value of 7.9 ± 0.9 nM that matched the reported values in the literature.[42] The *in vitro* activities of all compounds were measured at various concentrations (generally ranging from 10 nM to 3 μM) and inhibition curves for most of the tested analogs were obtained (**Figure 9**).

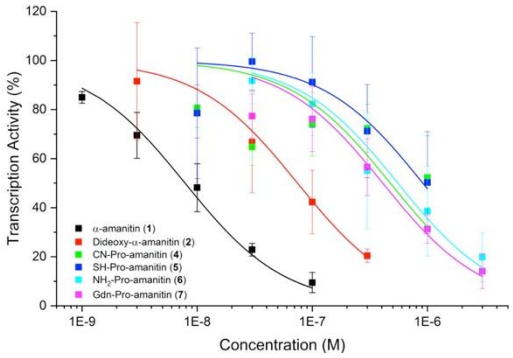

Figure 9. IC_{50} curves obtained for amanitin analogs CN, SH, NH_2, Gdn-AMA, dideoxy-α-AMA and α-amanitin in an *in vitro* transcription assay using the HeLa nuclear extract (n=3).

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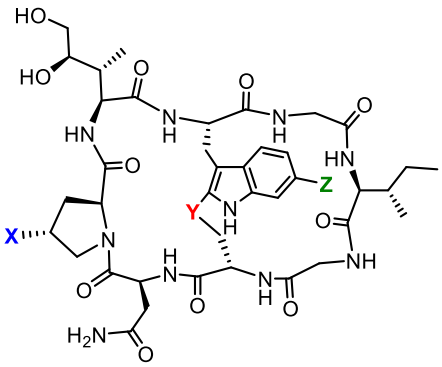
A full IC_{50} curve was not acquired for the analogs that did not exhibit any appreciable inhibitory effects up to 10 μM. The results for the transcription assay of amanitin analogs containing various proline residues are summarized in **Table 2.**

Table 2. Results of the *in vitro* transcription assay for α-amanitin and synthetic analogs using the HeLa nuclear extract (n=3). Note that the R^2 value for CN-Pro-AMA **4** shows an extremely poor fit for the IC_{50} curve.



Cmpd.	X	Y	Z	IC_{50} (nM)	R^2
1	OH	(*R*)-SO	OH	7.9 ± 0.90	0.991
2	OH	S	H	74.2 ± 5.4	0.980
3	N_3	S	H	> 1000	-
4	CN	S	H	480 ± 380	-5.760
5	SH	S	H	910 ± 230	0.860
6	NH_2	S	H	550 ± 90	0.983
7	Gdn	S	H	410 ± 80	0.918
8	SAcm	S	H	> 1000	-
9	Boc-Gdn	S	H	> 600	-
32	MeOCONH	S	H	N/A	N/A

Of all synthetic analogs, dideoxy-amanitin **2** was the most active inhibitor with an IC_{50} value of 74 ± 5 nM; the loss of activity compared to the natural product is most likely understood in terms of the loss of the H-bonds formed with the 6'-OH and/or a slightly weaker π-cation interaction owing to an indole ring with lower electron density. An explanation for its near-native cytotoxicity may yet be due to fact that RNA Pol II is highly expressed and may attain concentrations as high at 500 nM, a concentration that is higher than the Kd of **2**.

Cpd **7** (guanidine) shows an IC_{50} value of 410 ± 80 nM, which is approximately 50-fold higher than that of the natural product and yet only 6-fold less active than **2**. Other potentially active analogs include amino-proline **6** (IC_{50} = 550 ± 90 nM) and mercapto-proline **5** (IC_{50} = 910 ± 230 nM). Analogs that did not exhibit any noticeable inhibitory effects up to 1 μM concentrations include N_3-, SAcm- and Boc-guanidino-Pro amanitins. All compounds were investigated three times (n=3) except for 4 (n=4), which proved puzzling in terms of its inhibitory effects on Pol II leading to an unexplained but reproducible inability to fit an IC_{50} curve to the data points making curve-fitting especially difficult and leading us to question the IC_{50}-value that we obtained for this analog (**Figure 9**). Based on the transcription results for the mercapto-proline analog, IC_{50} value of 910 ± 230 nM (R^2 = 0.860), we cannot completely exclude the possibility of disulfide formation to give dimers of **5**. Nevertheless, we note that **5** was resynthesized and immediately subjected to the transcription assay in buffer supplemented with DTT, which likely would have reduced any disulfide bonds that might have formed.

To investigate the possibility of using synthetic amanitin analogs as ADC payloads, two analogs were selected for incorporation into HER2-targeting ADCs: CN-Pro (**4**) and NH_2-Pro (**6**). In both cases, a maleimide-based Val-Ala linker with a para-aminobenzyl-based conjugation site was employed (**Figure 10**). Two different linkers were adopted to address the versatility of linking strategies; in the case of **6**, we exploited the masking of the amino proline with an immolative *p*-aminobenzylcarbamate while in the case of **4**, we applied a *p*-aminobenzyldioxolane formed from the DHIle; upon enzymatic cleavage, the *p*-aminobenzyldioxolane is known to immolate to give the diol as previously reported.[43]

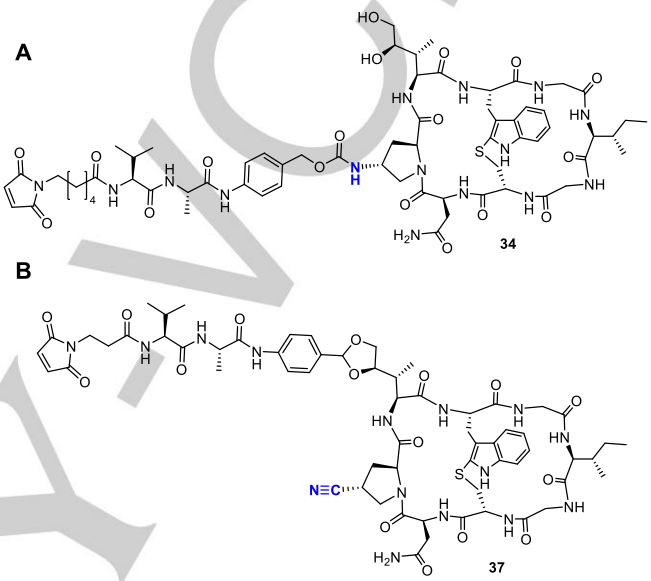


Figure 10. Chemical structure of synthetic ADCs of: (**A**) NH_2-Pro-AMA analog (**34**), and (**B**) CN-Pro-AMA analog (**37**). A maleimide Val-Ala-based linker has been used in both cases (see Supporting Information for reagents and reaction conditions).

In the case of **6**, the amine group on NH_2-Pro was conveniently used to attach the linker as a carbamate (**Figure 10**, A). However, to install said linker on CN-Pro-amanitin, the hydroxyl groups of the DHIle residue were protected by cyclic acetal formation with the *para*-aminobenaldehyde terminus (**Figure 10**, B). Both linker-toxin intermediates were site-specifically conjugated to an anti-HER2 antibody (T-D265C thiomab, generated by Heidelberg Pharma Research GmbH) to furnish the corresponding ADCs (see Supporting Information). The resulting ADCs were subjected to *in vitro* cell-based assays against three $HER2^+$ cell lines (SK-BR-3, SK-OV-3 and JIMT-1) and a $HER2^-$ cell line (MDA-MB-231) (**Figure 11**). Interestingly, while the antibody conjugates against these two analogs did not show detectable cytotoxicity against SK-OV-3 and JIMT-1 or MDA-MB-231, there was significant toxicity against SK-BR-3. As the SK-BR-3 have the highest expression of HER-2, they are likely to be more sensitive to otherwise less toxic payloads.

Of note, this result demonstrates the potential for designing amanitin analogs that may have specific toxicity against certain cell lines and not others.

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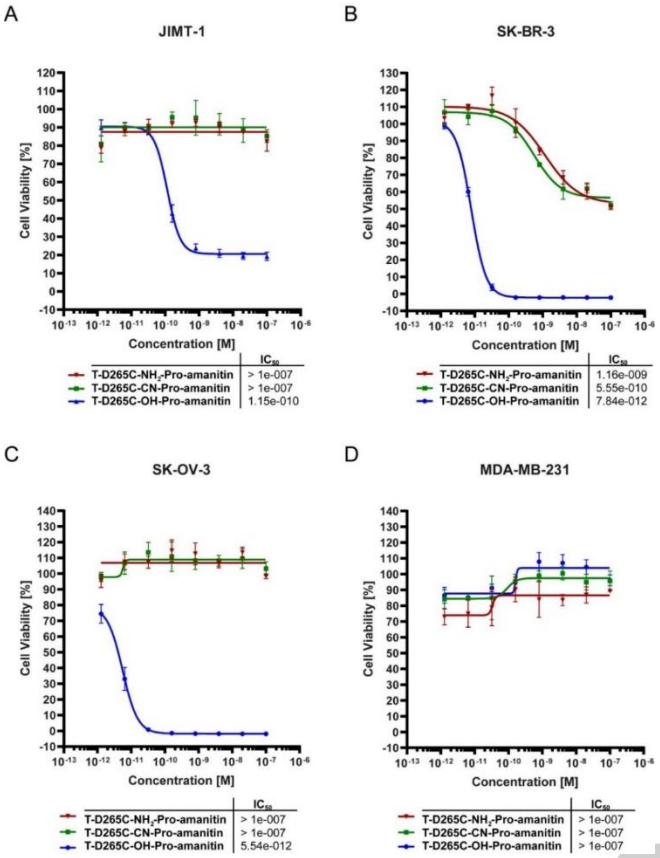

Figure 11. Results of the *in vitro* cell-based assay of anti-HER2 ADCs of CN-Pro- and NH_2-Pro-amanitins against (**A**) JIMT-1 ($HER2^+$), (**B**) SK-BR-3 ($HER2^+$), (**C**) SK-OV-3 ($HER2^+$), and (**D**) MDA-MB-231 ($HER2^-$) cells.

Among the Hyp analogs incorporated into the dideoxy-amanitin core, surprisingly none was as active as dideoxy-amanitin (**2**), as assayed in cytotoxicity assays and by run-off *in vitro* transcription assays. This disparity between the hydroxyproline and known analogs is challenging to rationalize and merits further discussion.

Although MMFF-based molecular modeling (Spartan, data not shown) suggested varying degrees of success with all of these derivatives, we recognized the challenge of replacing a (4*R*)-hydroxyl group on proline with a hydrogen-bond donating/accepting group that would enforce the C_γ-*exo* conformation.

CD spectra suggest that the overall 3D structures of the synthetic analogs differ slightly from dideoxy amanitin, but generally resemble each other (**Figure 7**, A). Alpha-amanitin (**1**) and all synthetic analogs show a positive Cotton effect past 250 nm, yet behave differently at wavelengths below 250 nm with the synthetic analogs exhibiting a local minimum at 230-234 nm, while α-amanitin shows a local minimum at 220 nm. The difference between the synthetic toxins and α-amanitin may be explained by the fact that α-amanitin (**1**) has a different chromophore owing to the 6'-OH. Greater differences were observed when the CD-spectra were acquired at pH 4.5. Although the relevance of working at pH 4.5 could be questioned, we chose this to complement our study in an attempt to find a medium where differences could be revealed. However, it is not readily understood why changing the pH would cause such dramatic deviation since none of the prolines has an ionizable group that would be perturbed upon decreasing the pH from neutrality to 4.5.

While CD is used extensively to characterize amanitins and many other peptides, we caution that CD is qualitative at best for inferring meaningful structural differences that would inform bioactivity; case in point: amanitin-sulfoxide analogs with very disparate CD spectra are found to be very structurally similar.[43] Indeed, of several amanitins that have been crystallized, these show nearly superimposable structures despite exhibiting significantly different CD spectra and equally disparate cytotoxicity IC_{50} values.[15c]

We next considered the putative H-bond interactions with Pol II. To date, cryo-EM and XRD structures have not directly observed the toxin at sufficient resolution to unequivocally reveal specific H-bonds and instead have been refined in accord with the ground-state structure determined by Lipscomb and coworkers for β-amanitin.[15a, b] As shown by Kornberg[10, 44] and Cramer[11-12, 45] in several reports, the most notable interaction of α-amanitin with Pol II is an H-bond between the hydroxyl group of Hyp and the glutamic acid residue (A845 Glu) of Pol II (**Figures 1** and **12**).

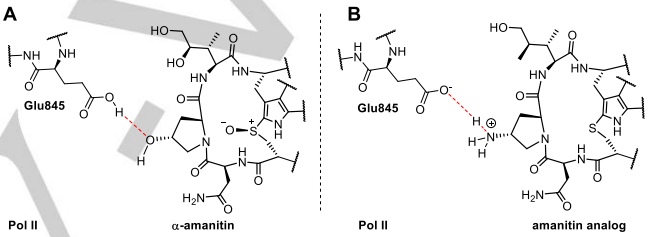

Figure 12. (**A**) Observed H-bonding interactions (red lines) between the hydroxyl group of Hyp in α-amanitin and Pol II from *S. scrofa*. The oxygen of the OH can accept a proton from Glu845 and the proton of the OH could act as an H-bond donor although the acceptor is currently unknown. (**B**) Expected interactions between the ammonium group of NH_2-Pro-AMA analog and Pol II. A proton from the NH_3^+ group can form a salt bridge with Glu845, sill still providing an H-bond donor. Similarly, a bifurcated set of H-bonds could be envisioned for the Gdn-Pro-AMA analog (not shown).

Nevertheless, the net energetic contribution of these H-bonding interactions is likely low since the toxin must be desolvated from bulk solvent wherein the same H-bonds will exist. Notwithstanding the thermoneutrality of H-bonding, we reasoned that the (4*R*)-amino-, (4*R*)-guanidino-, (4*R*)-methylcarbamoyl-, and (4*R*)-cyano-proline could be recognized by Glu845, either through H-bonding, charge-charge complementarity, or both. Similarly, the methylcarbamoyl-proline proved inactive despite the strong potential for H-bonding and an expected C_γ-*exo* conformation. As a control, we used the (4*R*)-azido-proline that is incapable of H-bonding. The fact that **6** (ammonium) and **7** (guanidinium) were the strongest inhibitors of Pol II in this series may point to the potential for accessing the H-bonding interactions seen in the crystal and cryo-EM structures while assuming the correct C_γ-*exo* conformation.

We expected lower IC_{50} values for NH_2- and Gdn-Pro amanitins as we anticipated these to form H-bonds or salt bridges with Glu845 of Pol II. Whereas as cations (NH_3^+ and $GdnH^+$, respectively), these would not serve as H-bond acceptors, yet they could form strong salt bridges with Glu845 while still donating an H-bond to a putative acceptor. Protected versions including the *N*-Boc-protected guandidine-proline and *S*-Acm-protected mercapto-proline were also evaluated as these present additional functionalities capable of further H-bonding interactions that may

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find supplementary interactions within the binding site. Whereas we did not expect success with these protected analogs given their extra steric bulk, we felt their evaluation would complement this study.

With respect to the mercapto-prolyl-amanitin (**5**), we recognized that the thiol may serve as a possible analog of a hydroxyl group albeit being a poor H-bond donor and incapable of accepting an H-bond.[46] A preference for C_γ-*endo* ring puckering in this case may further explain the generally poor inhibitory activity of this analog but not the others that prefer the C_γ-*exo* conformation. Nevertheless, the energetic difference between the *exo* and *endo* forms is on the order of ca.-1 kcal/mol at 25°C, which translates to an *exo/endo* ratio of only *ca.* 6.[16c, 47] This conformational bias alone is not enough to explain why this analog is so much poorer an inhibitor compared to **2**. However the added differences in H-bonding capacity may further erode inhibitory activity. More challenging however is to rationalize why prolines that are properly biased in favor of the C_γ-*exo* conformation show such poor inhibitory activity and correlated cytotoxicity.

The *in vitro* toxicity of the synthetic analogs was further assessed on HEK293 (**Figure ESI.7** in Supporting Information) and in HEK293-OATP1B3 cells that overexpress the organic anion-transporting polypeptide 1B3 (OATP1B3) (**Figure ESI.8** in Supporting Information) to assess whether OATP1B3 mediates active transport of α-amanitin and the Hyp analogs of amanitin into the cell. Human embryonic kidney HEK293 cells lack the OATP1B3 transporter (*wt*-HEK293) and hence showed only micromolar susceptibility to α-amanitin (IC_{50} = 0.13 ± 0.08 μM) and β-amanitin (IC_{50} = 0.12 ± 0.09 μM), while the amanitin derivatives showed no cytotoxic effect. On the contrary, transfected HEK293 cells constitutively expressing OATP1B3 (HEK293-OATP1B3) showed clearly enhanced sensitivity to α-amanitin, with an IC_{50} value of 43 ± 7.4 nM. The 3-fold increase in toxicity of α-amanitin on HEK293-OATP1B3 cells relative to HEK293 cells indicates that this toxin is subject to OATP1B3-mediated transport (note β-amanitin – used as a positive control, owing to an anionic aspartate in lieu of the asparagine is an even better substrate for the OATP1B3 transporter as indicated by a 15-fold reduction of the IC_{50} value to 8.5 ± 1.1 nM).

These analogs showed no cytotoxicity on HEK293 cells and only a very mild cytotoxic effect only at high concentrations in the μM range on HEK293-OATP1B3 cells. For example, at the highest used concentration of 1 μM, cell viability of NH_2-Pro-amanitin showed the lowest value of 33%, in comparison to N_3-Pro-amanitin (68%), SH-Pro-amanitin (50%), CN-Pro-amanitin (43%), Gdn-Pro-amanitin (86%), MeOCONH-Pro-amanitin (61%), α-amanitin (0%) and β-amanitin (0%). This slightly lower cell viability of OATP1B3-overexpressing cells compared to HEK293 cells after exposure to Hyp analogs at identical concentrations could be an indication that these variants are only partially transported into the cell *via* the organic anion-transporting polypeptide 1B3 (OATP1B3). However, in direct comparison to α-amanitin, these variants exhibit substantially lower cytotoxicity on these overexpressing cells as the natural compound. This reduced cytotoxic potential could be attributed to (*i*) a reduced binding affinity to RNA Pol II and/or (*ii*) limited uptake into the cell *via* the OATP1B3 transporter. The correlation between the inhibitory effect on Pol II and the cytotoxic potency on HEK-OATP1B3 cells of the different Hyp analogs supports the assumption that Pol II binding affinity plays a decisive role in mediating cytotoxicity. This finding is in line with the absence of any cytotoxic effect on *wt*-HEK cells – assuming unspecific and thus comparable uptake of all variants into these cells lacking the OATP1B3 transporter.

The reduced yet detectable binding affinity of selected Hyp variants on Pol II, along with the considerably reduced cytotoxic effect on OATP1B3-overexpressing HEK293 cells, prompted us to develop ADCs based on specific variants for targeted delivery to cancer cells and controlled release of the payload through cleavage of the inter-positioned protease-sensitive linker. We selected NH_2-Pro-amanitin, which showed a strong inhibitory potential on Pol II, for attachment of a valine-alanine linker *via* carbamate linkage. Additionally, we modified CN-Pro-amanitin with this Val-Ala linker through a cyclic acetal (see Supporting Information). For PoC *in vitro* studies, these amanitin-linker variants derived from NH_2-Pro-amanitin and CN-Pro-amanitin were conjugated to Trastuzumab for targeted delivery to $HER2^+$ cells using site-specific conjugation chemistry.

In vitro characterization was performed on three $HER2^+$ cell lines (SK-BR-3, SK-OV-3 and JIMT-1) and on one $HER2^-$ ($HER2^{low}$) cell line (MDA-MB-231; **Figure 11**). The cytotoxic potency of the two ADCs with Hyp analogs was compared to an ADC with an α-amanitin variant as the cytotoxic payload using the same Val-Ala linker (T-D265C-OH-Pro-amanitin). All $HER2^+$ cell lines showed high sensitivity towards the ADC based on α-amanitin with IC_{50} values of 0.12 ± 0.01 nM for JIMT-1, 7.84 ± 0.14 pM for SK-BR-3 and 5.54 ± 0.71 pM for SK-OV-3 cells, respectively. In contrast, JIMT-1 and SK-OV-3 cells did not show any response towards the Hyp-amanitin variants (**Figure 11**, C). However, on SK-BR-3 cells (highest HER2-expression), the Hyp variants were able to induce a mild cytotoxic effect by reducing cell viability to approx. 50% with an IC_{50} value of 1.16 ± 0.89 nM for T-D265C-NH_2-Pro-amanitin, and an IC_{50} value of 0.55 ± 0.10 nM for T-D265C-CN-Pro-amanitin, respectively (**Figure 11**, C). The $HER2^-$ cell line MDA-MB-231 did not respond to ADC treatment indicating that no unspecific effects are responsible for the cytotoxicity on $HER2^+$ cell lines (**Figure 11**, D).

The reduced inhibitory activity of some of the modified amanitin derivatives nevertheless show the promising potential of these analogs for therapeutic applications if used as payload for ADCs. The combination of being a poor substrate for OATP1B3 transporters whilst retaining inhibitory activity of amanitin to a certain degree might help to develop ADCs with reduced payload-mediated toxicity and an improved target-specific effect. ADCs based on low potency payloads like SN38 (e.g. ENHERTU®) make use of high drug-antibody ratios to overcome the limitation of the payload. Notwithstanding that toxicity was greatly diminished, the aim of synthesis must be to address SARs. This work will inform the design of provide toxins with attenuated toxicity such that these modified derivatives may still serve as candidate payloads for the development of an ADC.

Conclusion

At this juncture, we can conclude that Hyp residue is essential for the cytotoxicity and *in vitro* inhibitory activity of α-amanitin and replacement with analogs that afford the same conformational C_γ-*exo* pucker of Hyp significantly erode toxicity. Surprisingly, all analogs were far less cytotoxic than α-amanitin and dideoxy-amanitin however when tested in vitro, two analogs (**6** & **7**) were only 5-fold less active than **2**. While an ^1H-^{15}N NMR study could provide more insight into conformational effects, intra-annular

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NOE distances are unlikely to differ by more than an angstrom and such would be difficult to accurately quantify. Furthermore, such would only describe an envelope of ground state structures and would not fully interrogate the bound form.

X-ray or cryo-EM structures of Pol-II/amanitin would be more informative, yet it is unlikely that these will provide the resolution needed. As the acquisition of such structural data goes far beyond the scope of this study, we cautiously assert that the effects of the proline substitution must induce subtle structural effects and/or mismatched interactions within the polymerase that conspire to effect significantly lower potency. Future studies to obtain structural information are anticipated to complement this work in defining the critical nature of the Hyp on cytotoxicity.

In conclusion, this is the first report to address the role of the Hyp in amanitin by testing analogs thereof, four of which were chosen for their well-known C_γ-*exo* conformation characteristic of Hyp while providing a test of H-bonds that engage the target RNA Pol II. These analogs failed to show significant toxicity highlighting the mystery that continues to shroud this venerated toxin. Replacing the hydroxyl group on Hyp with either an amine, guanidine, or thiol may yet provide useful chemical handles for the design of bio-reducible and self-immolating conjugates. If potency can be augmented by the use of a targeting agent such as an antibody, lower inherent cytotoxicity might actually prove advantageous for therapeutic applications, particularly if the less-potent composition provides a chemical handle for further mediating intracellular activation or reducing overall systemic toxicity. Hence these analogs may still find use in the design of new amanitin-bioconjugates for ADCs.

Conflict of Interest

Compositions of matter described herein are the subject of a PCT filing. Heidelberg Pharma GmBH holds patents on derivatives of amanitin and conjugates thereof.

Experimental Section

Experimental Details are given in the electronic supporting information.

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Keywords: amanitin • amatoxin • hydroxyproline • peptide • antibody-drug conjugate

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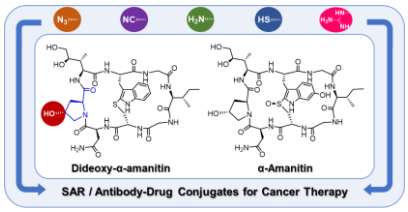

Getting at the Hyp of Amanitin: A formal study of structure-function relationships for trans-hydroxyproline – a key functionality the specifies the toxicity of amanitin is undertaken to probe aspects of H-bonding and other interactions with analogs of hydroxyl-proline. The toxin is surprisingly refractory to replacing the hydroxyproline.

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