Ether Derivatives of α-Amanitin. Introduction of Spacer Moieties, Lipophilic Residues, and Radioactive Labels†

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ABSTRACT: Etherification of α-amanitin with tritiated methyl iodide yielded a radioactively labeled amatoxin of high specific activity (~4 Ci/mmol) which, in its inhibition capacity for RNA polymerase II, was very similar to α-amanitin. The labeled toxin was used successfully in binding assays with RNA polymerases II and in radioimmunological determinations of amatoxins. If long-chained alkyl bromides were reacted with α-amanitin, lipophilic ether derivatives were obtained with a facilitated penetration capacity into cells. As a consequence of the improved permeability, two derivatives, *O*-hexyl- and *O*-decyl-α-amanitin, were more toxic in vivo than α-amanitin, although their affinity to RNA polymerases II was much reduced. By reaction of *N*-*tert*-butyloxycarbonyl-*N*′-(6-bromocaproyl)ethylenediamine with α-amanitin, a ten-atom spacer with a terminal amino group could be introduced into the toxin, which allowed the attachment of α-amanitin to proteins, solid-phase supports, or reporter groups. For example, by reaction with fluoresceinyl isothiocyanate, a fluorescent amatoxin was prepared for visualizing amatoxin-binding structures in cells. After succinylation of the spacer moiety, α-amanitin could be attached to proteins, e.g., fetuin, yielding a derivative with good antigenic properties. When an α-amanitin derivative was coupled to Sepharose 6B, an adsorbent for affinity chromatography was obtained suitable for a one-step purification of amatoxin-binding immunoglobulins from the sera of immunized rabbits.

Amatoxins inhibit eukaryotic RNA polymerase II (or B) at a toxin concentration of about 10^{-8} M while RNA polymerase III (or C) is 1000 times less sensitive and RNA polymerase I (or A) is resistant. The high specificity of amatoxins for the enzyme transcribing precursor mRNA has made them important tools of molecular biology.

Chemical modifications of the toxins were attempted either to learn about the relation of structure to biological activity or to introduce functional groups and labels into the toxin molecule [for a review, see Wieland & Faulstich (1978)]. So far, all these modifications were achieved at three side chains of the toxin, which are available for chemical reactions (Figure 1). These side chains include those of aspartic acid (position 1), dihydroxyisoleucine (position 3), and the 6′-hydroxylated tryptophan (position 4). The types of amatoxin derivatives prepared so far are compiled in Table I.

Most of the chemical modifications in the past made use of the naturally existing carboxylic group in β-amanitin. Various esters or amides of type **2**, including α-amanitin (**1**), have been prepared from β-amanitin by Wieland & Boehringer (1960). The reverse reaction, the deamidation of α-amanitin (**1**) to β-amanitin (**2a**), could not as yet be achieved (A. Buku, unpublished experiments).

Other modifications involved the side chain of the dihydroxylated isoleucine, which can be submitted to a glycol-splitting reaction with periodate (Wieland & Fahrmeir, 1970). The resulting aldo compound (**3a**) was devoid of toxicity but could be converted to the toxic *O*-methyl(dehydroxymethyl)-α-amanitin (**3b**) by hydrogenation. A disadvantage of this type of derivatization was that the hydroxylated isoleucine side chain is, without any doubt, part of the active site of the amatoxins (Cochet-Meilhac & Chambon, 1974). Thus, the lack of the γ-hydroxymethyl group decreases the toxicity of **3b** to 10% of that of α-amanitin. Nevertheless, tritiated **3b** has in the past been most widely used for biochemical studies (Cochet-Meilhac & Chambon, 1974).

Finally, some modifications made use of the 6′-hydroxyindole moiety of the amatoxins. In one type of reaction, the aromatic part was shown to be accessible to electrophilic substitutions, e.g., with diazonium ions (Faulstich & Trischmann, 1973; Hencin & Preston, 1979). Unfortunately, the azo linkage in derivatives of type **4** connecting the spacer moiety with the toxin must be expected to be unstable under physiological conditions (Trefouel et al., 1935). By another electrophilic substitution of the indole nucleus, Morris et al. (1978) achieved the iodination of α-amanitin with iodine monochloride in the 7′ position. In another type of reaction, the phenolic hydroxy group of the indole moiety can be converted to ether derivatives of amatoxins. The methyl ether (**5a**) of α-amanitin can be obtained from **1** by reaction with either diazomethane or methyl iodide. This compound exhibits the same toxicity as that of the parent compound. The same was true for *O*-methyl-γ-amanitin (Gowindan, 1969), which was the first radioactivity labeled amatoxin used in biological experiments (Meilhac et al., 1970).

After many discouraging attempts to introduce distinct residues into β-amanitin, we finally studied the derivatization of α-amanitin by etherification. The procedure, as well as the physical and the biological properties of 13 new 6′-ether derivatives of α-amanitin, will be presented in this paper (Table II, **5c–5o**, **6**).

Materials and Methods

Reagents. α-Amanitin (**1**) was a preparation of our laboratory. Methyl iodide, ethyl iodide, propyl iodide, allyl bromide, hexyl bromide, decyl bromide, benzyl bromide, bromoacetone, and 6-bromocaproic acid were from Merck (Schuchardt), Darmstadt, West Germany. [^{3}H]Methyl iodide (specific activity 4 Ci/mmol) was from NEN, Dreieich, West Germany.

Ethers of α-Amanitin. In a typical reaction, 50 mg (55 mol) of **1** was dissolved in 2 mL of dry ethanol. In another vessel, 10 mg of sodium was dissolved in 20 mL of dry ethanol, and 2.4 mL (60 mol = 1.1 equiv) of the sodium ethylate solution was added to the toxin. The reaction mixture was immediately evaporated in vacuo at 30 °C, and the residue was dissolved in 2 mL of dry dimethylformamide and reacted with 2.5–4.0

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Table I: Review of the Structure, Toxicity, and RNA Polymerase II Inhibition Capacity of Several Derivatives of α- and β-Amanitin[a]

no.	side chains	compd	toxicity (mg/kg)	toxicity (mg of α-amanitin/kg)	inhibition capacity (α-amanitin = 100)	ref
1		α-amanitin	0.3	0.3	100	
2a	$R^I = OH$	β-amanitin	0.4	0.4	92	
2b	$R^I = OCH_3$	β-amanitin methyl ester	0.8		17	
2c	$R^I = SC_6H_5$	β-amanitin thiophenyl ester	0.6	0.5		Wieland & Boehringer
2d	$R^I = NHC_6H_5$	β-amanitin anilide	>4.5	>4.2	17	(1960)
2e	$R^I = NHC_{12}H_{25}$	β-amanitin dodecylamide	>4	>3.4		
3a	$R^{II} = CHO$; $R^V = CH_3$	*O*-methyl-*aldo*-α-amanitin	>20	>20	<2	Wieland & Fahrmeir
3b	$R^{II} = CH_2OH$; $R^V = CH_3$	*O*-methyl(dehydroxymethyl)-α-amanitin[b]	3.0	3.0	48	(1970)
4a	R^{III} = H·TFA	[4′-[[[(6-aminohexyl)amino]carbonyl]phenyl]azo-α-amanitin trifluoroacetate	1.2	0.9	22	Faulstich & Trischmann (1973)
4b	$R^{III} = COOC(CH_3)_3$	[4′-[[[6-[(*tert*-butyloxycarbonyl)-amino]hexyl]amino]carbonyl]-phenyl]azo-α-amanitin				
4c	$R^{IV} = I$	7′-iodo-α-amanitin			>100	Morris et al. (1978)
5a	$R^V = CH_3$	*O*-methyl-α-amanitin	0.3	0.3	85	
5b	$R^V = C_6H_4CN_4H$	*O*-(4′-tetrazolyl)phenyl-α-amanitin	0.3	0.25		Buku & Wieland (1975)

[a] Toxicity is expressed as LD_{50} values in milligrams of derivative or α-amanitin, respectively, per kilogram of body weight of the white mouse. Inhibition of RNA polymerase is expressed as the percent inhibition capacity of calf thymus RNA polymerase II related to the 50% inhibition value of α-amanitin, which is equal to 100. [b] Former name, *O*-methyldemethyl-γ-amanitin.

Table II: Structure, Toxicity, and RNA Polymerase II Inhibition Capacity of Ether Derivatives of α-Amanitin[a]

no.	side chains	compd	yield of the etherification reaction (%)	yield of other reactions (%)	toxicity (mg/kg)	toxicity (mg of α-amanitin/kg)	inhibition capacity (α-amanitin = 100)
5a	$R^V = CH_3$	*O*-methyl-α-amanitin	30		0.3	0.3	85
5c	$R^V = C_2H_5$	*O*-ethyl-α-amanitin	28		2.4	2.3	80
5d	$R^V = C_3H_7$	*O*-propyl-α-amanitin	40		6.0	5.8	60
5e	$R^V = C_3H_5$	*O*-allyl-α-amanitin	38		2.8	2.7	63
5f	$R^V = C_6H_{13}$	*O*-(*n*-hexyl)-α-amanitin	30		0.25	0.2	20
5g	$R^V = C_{10}H_{21}$	*O*-(*n*-decyl)-α-amanitin	26		0.32	0.3	24
5h	$R^V = CH_2C_6H_5$	*O*-benzyl-α-amanitin	22		1.8	1.6	13
5i	$R^V = CH_2COCH_3$	*O*-acetonyl-α-amanitin	30		3.5	3.3	20
5k	$R^V = CH_2CH(OH)CH_3$	*O*-(2-hydroxypropyl)-α-amanitin		71	>5.0	>4.7	3
5l	$R^V = (CH_2)_5COOH$	*O*-(5-carboxypentyl)-α-amanitin	30		1.1	1.0	27
5m	$R^V = (CH_2)_5CONH(CH_2)_2NH_2$·TFA	*O*-[5-[[(aminoethyl)-amino]carbonyl]-pent-1-yl]-α-amanitin trifluoroacetate		95	>10	>7.8	36
5n	$R^V = (CH_2)_5CONH(CH_2)_2NHCO(CH_2)_2COOH$	*O*-[5-[[[(succinoyl-amino)ethyl]-amino]carbonyl]-pent-1-yl]-α-amanitin		60			22
5o	$R^V = (CH_2)_5CONH(CH_2)_2NHCOOC(CH_3)_3$	*O*-[5-[[[β-[(*tert*-butyloxycarbonyl)-amino]ethyl]-amino]carbonyl]-pent-1-yl]-α-amanitin	46		1.8	1.4	25
6	$R^V = CH_3$; $R^{VI} = CH_3$	*O*,*N*-dimethyl-α-amanitin	10				53

[a] Toxicity and inhibition capacity are expressed as in Table I, except for the enzyme, where RNA polymerase II of *Drosophila melanogaster* embryos was used (α-amanitin ≡ 100).

equiv of the alkyl halogenide for 12 h at 20 °C.

After evaporation of the solvent in vacuo, the reaction mixture was applied to three HF_{254} thin-layer silica plates (Merck, analytical type) and developed with chloroform/methanol/water (65:25:4). The three strongest bands as identified under UV light were immediately scraped off the plate. In the order of ascension on the plate, the compounds were dialkyl-α-amanitin, alkyl-α-amanitin, and then unreacted α-amanitin. The silica powder was instantly eluted with methanol and then filtered, and the solvent was evaporated in vacuo. Residual silica was separated by chromatography on LH20 columns (90 × 1.8 cm) developed with methanol. Elution was monitored by the OD at 282 nm. In most cases, a single symmetric peak was obtained. Yields were the fol-

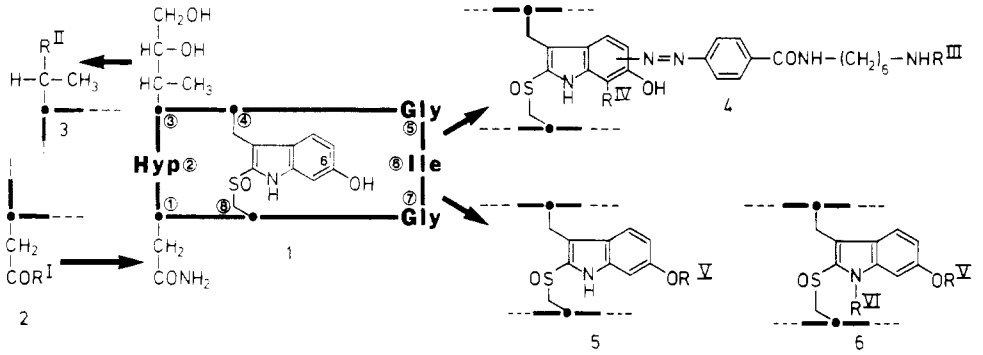

FIGURE 1: Scheme of all derivatization reactions of α- and β-amanitin performed in our laboratory: **1**, α-amanitin; **2**, β-amanitin and derivatives; **3**, derivatives of α-amanitin obtained by periodate degradation of the hydroxylated isoleucine side chain; **4**, azo derivatives of α-amanitin; **5**, monoalkylated derivatives of α-amanitin; **6**, dialkylated derivatives of α-amanitin.

lowing: alkyl-α-amanitin, 22–49% dialkyl-α-amanitin, 5–9%; α-amanitin (recovered) 26–30%. The alkylated derivatives were pure as shown by thin-layer chromatography (TLC) in *sec*-butyl alcohol/ethyl acetate/water (14:12:5) or chloroform/methanol/water (65:25:4). For removal of the *tert*-butyloxycarbonyl (Boc) blocking group, **5m** was treated with trifluoroacetic acid for 2 min and evaporated immediately. For removal of residual acid, the solid was digested several times with dry and peroxide-free ether.

Boc-ethylenediamine hydrochloride was prepared according to Geiger & König (1971). Purification was by crystallization, yield 30%.

N-Boc-N'-(6-bromocaproyl)ethylenediamine was synthesized via the mixed anhydride method. 6-Bromocaproic acid (195 mg, 1 mmol) was dissolved in 7 mL of dry tetrahydrofuran, together with 101 mg of triethylamine (1 mmol), cooled to 15 °C, and reacted with 108 mg of ethoxycarbonyl chloride (1 mmol) for 5 min. Boc-ethylenediamine hydrochloride (196 mg, 1 mmol), together with 101 mg of triethylamine in 10 mL of tetrahydrofuran, was added, and the mixture was allowed to come to 0 °C. It was kept at this temperature for 10 min, and then, after the addition of 1 mL of H_2O, it was kept at 20 °C for an additional 10 min. After evaporation in vacuo, the residue was dissolved in ethyl acetate and washed 3 times with citric acid (5%, cold), 3 times with potassium hydrogen carbonate (10%), and 3 times with sodium chloride (10%). Crystals formed in methanol/ethyl acetate/benzine (40 °C) with a yield of 74% had a mp of 102–103 °C, M_r 337.26. Anal. Calcd for $C_{13}H_{25}N_2O_3Br$: C, 46.3; H, 7.4; N, 8.3. Found: C, 47.0; H, 7.5; N, 8.2.

O-(2-Hydroxypropyl)-α-amanitin (**5k**). **5i** (14 mg) was dissolved in 1.2 mL of H_2O and reacted with 5.5 mg of sodium borohydride in 1.2 mL of methanol for 1 h at 20 °C. After that time, another 10 mg of sodium borohydride was added and the reaction continued for an additional 12 h at 4 °C. After acidification with acetic acid (pH 3–4), the solvent was evaporated in vacuo and the residue purified on TLC and LH20 Sephadex as described above. The yield was 71%.

O-[5-[[[(Succinoylamino)ethyl]amino]carbonyl]pent-1-yl]-α-amanitin (**5n**). **5o** (60 mg) was treated with 5 mL of trifluoroacetic acid for 2 min. Thereafter, the acid was removed in vacuo; residual acid was removed by three repeated evaporations in 10 mL of dry, peroxide-free ether. The residue was dissolved in water, adjusted to pH 8 with 0.1 N NaOH, and reacted at constant pH (8.5). After the addition of the reagent, the mixture was further reacted for 2–3 h at the same pH. The major part of the solvent was evaporated in vacuo, and the residual solution was applied to a column of Sephadex LH20 (220 × 2.5 cm) developed with methanol/water (1:1). The yield was 75%. In cases when the separation of **5o** from succinic acid was incomplete, the acid was separated on DEAE A50 ion-exchange column (23 × 3.2 cm) and eluted with a gradient of acetic acid, with an initial concentration of 0.01%. **5o** was eluted at 0.03% acetic acid.

Binding assays of radioactively labeled toxins were modified from the description of Cochet-Meilhac & Chambon (1974) and Cochet-Meilhac et al. (1974). Samples of RNA polymerase solution, as obtained after the DEAE-cellulose purification step (100 μL), were incubated with varying amounts of labeled toxin for 12 h at 20 °C in buffer I [0.15 M Tris-HCl, 0.1 mM dithioerythritol (DTE), and 35% (v/v) glycerol, pH 7.9]. After equilibration, the mixture was filtered through 2.5-cm filters of nitrocellulose (HAWP Millipore, 0.45 μm). The filters were washed 3 times with 10 mL of buffer II [0.1 M Tris-HCl, 0.1 mM EDTA, 0.1 mM DTE, and 1.5% (v/v) dimethyl sulfoxide (Me_2SO)] and counted in 10 mL of Bray's scintillation liquid. For the detemination of radioactivity which was unspecifically bound to the filters, the RNA polymerase solution was preincubated with 10 μg (100-fold excess) of nonlabeled α-amanitin before addition of the labeled toxin. The background value determined for a 4×10^{-2} μg/mL concentration of labeled toxin was 200 cpm.

Results

By the described alkylation procedure, 13 amatoxin derivatives were synthesized as compiled in Table II. The yield was 22–46%, even with complex alkyl bromides. Under the given conditions, the alkylation of the amatoxins occurred predominantly at the phenolic hydroxy group. Alkylation of this hydroxy group was proven by the absence of a phenolate spectrum. Such a spectrum is known for α-amanitin, which upon the addition of alkali is shifted by more than 20 nm to longer wavelengths.

After isolation and purification, the main reaction product of each alklation procedure was identified as the O-monoalkylated cyclopeptide. Proof for such monoalkylation was shown in two ways. First, amino acid analysis never detected any alkylated amino acids. Second, a reaction with tritiated methyl iodide (3.8 Ci/mmol) yielded *O*-methyl-α-amanitin of exactly the same specific activity.

Beside the monoalkylated toxins, small amounts of higher akylated derivatives of α-amanitin were found in each alkylation reaction. The main side product was, in all cases, the dialkylated toxin. In three cases, the dialkylated α-amanitin was isolated (5–10% yield). For example [^{3}H]dimethyl-α-amanitin was obtained in 10% yield with a specific activity of 7.3 Ci/mmol, demonstrating the stoichiometry of the reaction.

The second alkyl group introduced was located at the indole nitrogen. This was suggested by the small shift (~3.5 nm to

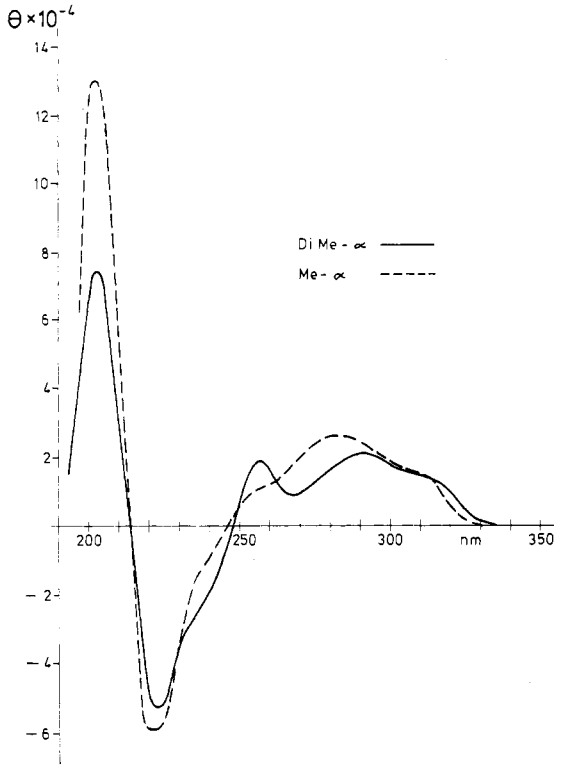

FIGURE 2: CD spectra of *O*-methyl-α-amanitin (dashed line) and *O,N*-dimethyl-α-amanitin (solid line), pH 7.0, in water.

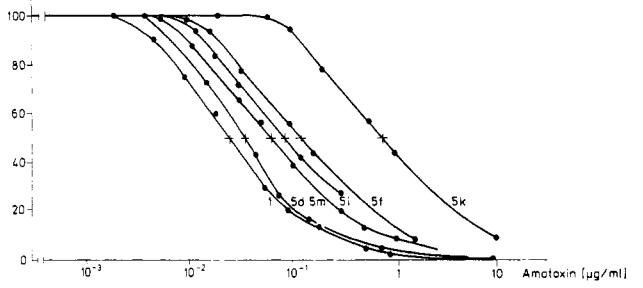

FIGURE 3: Dose–inhibition curves of several monoalkyl ethers of α-amanitin: **1**, α-amanitin **5d**, *O*-propyl-α-amanitin; **5m**, *O*-[5-[[(aminoethyl)amino]carbonyl]pent-1-yl]-α-amanitin; **5i**, *O*-acetonyl-α-amanitin; **5f**, *O*-hexyl-α-amanitin; **5k**, *O*-(2-hydroxypropyl)-α-amanitin.

longer wavelengths) in the UV spectrum of dialkylated toxins compared to those of α-amanitin and monoalkylated α-amanitin. The involvement of the indole nucleus in the second alkylation became more evident in the circular dichroism spectra, where the spectrum of the dialkylated α-amanitin is distinctly different from that of monoalkylated α-amanitin (Figure 2). There are only minor differences in the CD spectra of monoalkylated α-amanitin and α-amanitin itself (spectrum not shown).

All monoalkylated amatoxins inhibited the RNA polymerase II (or B) isolated from *Drosophila melanogaster* embryos (Greenleaf & Bautz, 1975). The inhibition capacities varied from 85 to 3% of that of the parent compound. Some of the dose–inhibition curves are shown in Figure 3. The 50% inhibition values (Table II) were, in all cases, related to α-amanitin (=100%) as an internal standard. This standard allows the comparison of the results of previous assays using calf-thymus RNA polymerase II (Table I) with the various ethers of α-amanitin in Table II.

Since monomethylated and dimethylated α-amanitin (**5a** and **6a**) were prepared with tritium labels, they could be used in binding assays. These results are depicted in Figure 4. The

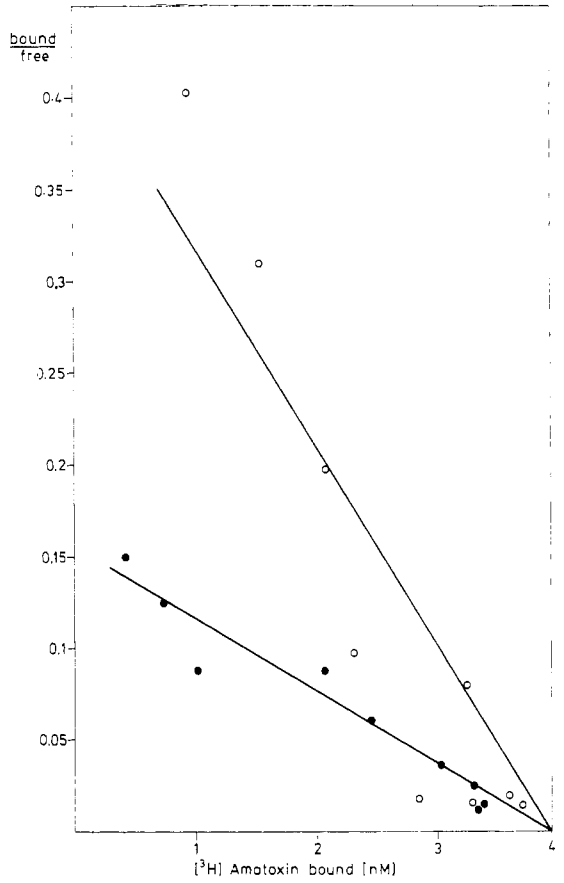

FIGURE 4: Scatchard plots of binding experiments of RNA polymerase II of *Drosophila melanogaster* embryos with [³H]methyl-α-amanitin (open circules) and [³H]dimethyl-α-amanitin (closed circles).

monomethylated amanitin and the dimethylated α-amanitin have K_A values of 1.1×10^8 and 3.9×10^7 M^{-1}, respectively. Accordingly, the introduction of the second methyl group at the indole nitrogen lowers the affinity to the enzyme by a factor of about 2.8.

Most of the monoalkylated amatoxins had strongly reduced toxicity in the white mouse, when compared to that of α-amanitin. Only three compounds, those with methyl, hexyl, and decyl residues, were as toxic or even more toxic than the parent compound. Methyl-α-amanitin is physically very similar to α-amanitin and consequently has very similar affinity (Figure 4) and inhibition capacity (Table II) for RNA polymerase II. Accordingly, the in vivo toxicity is also in the range of that of α-amanitin. The similarity confirms that the phenolic hydroxy group is not involved in the inhibition mechanism.

In contrast, the inhibition capacity of the two long-chain alkyl derivatives (**5f** and **5g**) was decreased to about 20% of that of α-amanitin (Table II). Remarkably, there was not a corresponding loss in toxicity. Rather, the toxicity of hexyl-α-amanitin was somewhat higher than that of α-amanitin. We conclude that for these two alkyl derivatives the permeability into cells must have changed by the introduction of the long hydrocarbon chains (see Discussion). The derivative with a terminal amino group (**5m**) was nontoxic up to 35-fold of the LD_{50} value of α-amanitin. The LD_{50} values of all derivatives in milligrams per kilogram of body weight (and reduced to their content of α-amanitin in milligrams of α-amanitin per kilogram of body weight) are compiled in Table II.

Another compound, with very reduced toxicity, was 2-hydroxypropyl-α-amanitin (**5k**). Since this compound also possesses the lowest inhibitory capacity against RNA polymerase II, different from that of the two other compounds with

C_3 residues (**5d** and **5i**), a conformational change was expected to exist, induced by the 2-hydroxy group in the ether moiety. Therefore, a CD analysis of **5k** was performed by using the acetonyl and propyl derivatives (**5d** and **5i**) for comparison. Unexpectedly, the CD curves of all compounds were very similar and, in turn, very similar to those of methyl-α-amanitin (**5a**) and α-amanitin (**1**).

Discussion

(*1*) *Previous Attempts for the Modification of Side Chains in Amatoxins.* Several efforts have been made for modifying amatoxins via their aliphatic side chains. Such attempts remained unsatisfactory because one of the side chains accessible to chemical reactions, that of dihydroxyisoleucine, turned out to be part of the active site. Another one, the carboxylic group of aspartic acid in β-amanitin, reacted rather slowly, with complex reagents in particular. This may be understood from the models of the three-dimensional structures of the amatoxins and, in comparison, of the phallotoxins, as established by X-ray analysis (Kostansek et al., 1977) or NMR analysis (Tonelli et al., 1978). While in phallacidin, the carboxylic group of D-β-hydroxyaspartic acid is exposed (Patel et al., 1973), that of aspartic acid in β-amanitin is folded inward and probably hydrogen bonded to an amide group of the peptide ring.

Better results than those obtained with aliphatic side chains were obtained when the aromatic part of the toxin was modified. In the supposed conformation, the indole nucleus is exposed from the molecule, but in contrast to the phallotoxins, the nucleus does not appear to be part of the toxin's binding site. As expected from the exposed position of the aromatic side chain, even complex moieties could be introduced as diazonium salts (Faulstich & Trischmann, 1973). However, the resulting azo compounds are probably sensitive to biological reducing agents as observed for prontosil rubrum, a sulfonamide containing an azo moiety (Trefouel et al., 1935). Products superior to the azo compounds were expected to be obtained by modification of the 6′-hydroxy group of the aromatic side chain. Therefore, this type of reaction was studied in some detail.

(*2*) *Etherification.* Ether derivatives of α-amanitin were, infact, obtained in 20–46% yield. This was also the case when complex alkyl bromides were reacted. Even the presence of functional groups in the alkyl bromides such as carbonyl (**5i**) or carboxyl (**5l**) did not seriously decrease the yield of the condensation reactions. For example, bromoacetone as well as sodium ω-bromocaproate yielded about 30% of the monoalkylated derivatives of α-amanitin. Besides the monoalkylated toxins, 5–10% of dialkylated peptides and about 30% of the starting material were isolated. Altogether, the yield of defined pure products was 70–80%, which, with regard to the complexity of some reactants, is very reasonable. Alkylation seems to be rather specific for the O atom of the aromatic ring and, to a lesser extent, for the indole nitrogen. By reaction with the strong base $C_2H_5O^-$, deprotonation of the two heteroatoms at the indole part can, in fact, be expected. However, to a small extent, one or the other amide proton was also abstracted, resulting in small amounts of compounds most likely alkylated in the N^{α} position of the amino acids. Such N^{α}-alkylation was not detected in the purified ether derivatives of α-amanitin.

(*3*) *Purification Procedures.* An excellent purification was achieved by using the analytical TLC procedure on a preparative scale. About 20 mg of the crude material could be applied to one thin-layer plate (0.05-cm layers of silica). With a suitable solvent system composed of chloroform, methanol, and water, a rapid and very efficient resolution of the product mixture was achieved. However, care must be taken to instantly remove that part of the silica layer containing amatoxins and elute it with methanol. Storage of the plate in the presence of light and air rapidly damages the toxins adsorbed to silica. After filtration of the residue over a short column of LH20 Sephadex, to remove residual silica, the products were pure by TLC in two solvent systems and by amino acid analysis. As lyophilized powder, all products were stable for several months at 4 °C.

(*4*) *Dialkylation.* Some derivatives of α-amanitin with two alkyl residues were isolated and characterized. Such derivatives were of some interest because two alkyl residues, at least of short hydrocarbon chains, did not significantly alter the biological properties of the compound. In all cases, the dialkylated amatoxins were easily distinguished on TLC by their polarity, which was less than that of the monoalkylated toxins. The second residue was found to be linked to the indole nitrogen atom, as proved by the UV absorption spectrum and the CD spectrum of dialkylated amatoxins, which differed from those of α-amanitin and its monoalkylated derivatives (e.g., see Figure 2).

In general, the alkylation at the indole nitrogen affects the biological activities of amatoxins to a lesser extent than the corresponding reaction in the phallotoxin series. In both toxin families, the introduction of one C atom only slightly decreases toxicity (Faulstich & Wieland, 1971) and the affinities to the target proteins, actin (H. Faulstich, S. Zobeley, and H. Trischmann, unpublished experiments) and RNA polymerase II. For example, *N*-methylphalloidin, *O*-methyl-α-amanitin, and *O*,*N*-dimethyl-α-amanitin have 90%, 85%, or 53%, respectively, of the binding (or inhibition) capacities of the parent compounds. However, the introduction of C_2, C_3 residues into phalloidin is deleterious to its toxicity and binding to actin (*N*-ethylphalloidin, 22%; *N*-propylphalloidin, 0% of phalloidin), while the effect of such substitutions on α-amanitin is small (*O*-ethyl-α-amanitin, 80%: *O*-propyl-α-aminitin, 60%; *O*,*N*-diethyl-α-amanitin, 50%). These data confirm that for the phallotoxins the indole nucleus is part of the peptide's binding site to the receptor protein (Wieland & Faulstich, 1979), while for the amatoxins it is not.

(*5*) *Radioactive Labels.* One aim of this study was to find an elementary preparation of radioactively labeled amatoxins, preferably by methylation, because methylated α-amanitin is close to the natural compound. In fact, 6′-methyl-α-amanitin can be easily prepared by reaction with diazomethane (Wieland et al., 1941). However, handling of tritiated diazomethane was to be avoided. Therefore, the methylation with methyl iodide was studied, which also represented the model reaction for other complex alkyl bromides.

[^{3}H]Methyl-α-amanitin of high specific activity could be prepared by using [^{3}H]methyl iodide and the sodium salt of α-amanitin. The reaction also ran well if only 1–2 mg of α-aminitin was used. Since the affinity of the dimethylated side product was determined by Scatchard analysis to be only 2.8 times lower than that of methyl-α-aminitin, the reaction yielded another amatoxin with a tritium label of 2-fold higher specific activity. Both methylated and dimethylated amatoxins were used successfully in assays with RNA polymerase II and with amanitin-binding immunoglobulins (H. Faulstich, S. Zobeley, and H. Trischmann, unpublished experiments). With radioimmunoassays, we detected amatoxins in biological fluids in concentrations as low as 3 ng/mL. Useful as they may be in in vitro assays, there are some indications that alkylated amatoxins may become problematic when introduced into in vivo systems. Preliminary experiments with preparations of

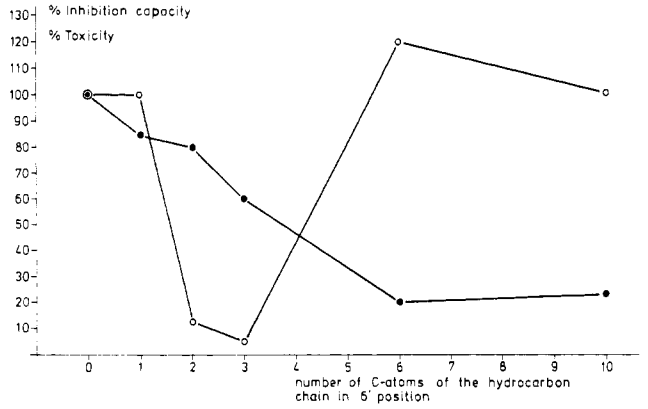

FIGURE 5: Inhibition capacity (for RNA polymerase II of *Drosophila melanogaster* embryos, closed circles) and in vivo toxicity (LD_{50} in white mouse, open circles) of several *O'*-alkyl-α-amanitins (number of carbon atoms in α-amanitin = 0).

liver microsomes demonstrated that methyl-α-amanitin, for example, undergoes at least partial oxidation with subsequent loss of the labeled residue.

(*6*) *Introduction of Lipophilic Residues.* Subsequent to the alkylation of amatoxins, we studied the introduction of lipophilic residues, which possibly would increase the permeability of the toxins through plasma membranes without seriously affecting biological activity. Such toxin derivatives, particularly with increased permeability for specific kinds of cells, are expected to be useful in cell biology. Penetration rates of the alkylated amatoxins into cells were not assayed directly but were estimated from their affinities to RNA polymerase II and their in vivo toxicities.

Binding to the enzyme decreases continuously with an increasing length of the hydrocarbon chain (C_1, C_2, C_3, and C_6 in Figure 5). However, the course of toxicity in the white mouse is completely different (Figure 5). The toxicity of the C_6 and C_{10} derivatives is equal to, or even higher than, that of α-amanitin.

The most reasonable explanation for this effect is that the lipophilic chains help the toxin to penetrate cells more easily. The actual amatoxin concentration in the affected cells must be about 5 times higher than that for α-amanitin, because the 80% decrease in enzyme inhibition of these compounds had been compensated or even overcompensated. It was not excluded, however, that the two derivatives with the facilitated penetration rate affect cells other than the parenchymal cells of liver and kidney, which are normally affected by α-amanitin. Accordingly, by the introduction of these hydrocarbon chains, the specificity of the toxin could have been changed. In addition, the steep decrease in toxicity measured after lengthening the chain by only one C atom (C_1 vs. C_2), which is not at all paralleled by the enzyme inhibition capacity, raises the possibility that target proteins other than RNA polymerase II (and with different binding properties) may be involved in the toxic activities of amatoxin.

(*7*) *Reduced Biological Activity Caused by Functional Groups of the Ether Moiety.* The compound with the lowest inhibition capacity for RNA polymerase II is the *O*-2-hydroxypropyl derivative of α-amanitin (**5k**). Its activity was determined to represent only 3% of that of α-amanitin, while other C_3 derivatives such as propyl-α-amanitin or acetonyl-α-amanitin have distinctly higher inhibition capacities, 60% and 20%, respectively.

We therefore investigated the compounds **5d**, **5i** and **5k** by circular dichroism, expecting evidence for a conformational change induced by an interaction between the hydroxy group of the moiety introduced and the amide bond of the amino acids in positions 4 and 5, according to the atomic model built after the X-ray analysis of Kostansek et al. (1977). However, the CD spectra of the acetonyl α-amanitin and the hydroxypropyl-α-amanitin were very similar to each other and also to the CD spectrum of α-amanitin (Figure 2). Hence, it is unlikely that a conformational change accounts for the loss of biological activity of compound **5k**. A more reasonable explanation may be that the new hydroxy group in **5k** interacts with an unknown structure of the enzyme surface, thereby disturbing the proper arrangement of the toxin–enzyme complex.

Another kind of decreased biological activity was determined for the derivative **5m**. Although this compound inhibits RNA polymerase II strongly, and, actually, to a higher extent than that expected from the length of the residue, the compound is nontoxic with the 30-fold LD_{50} of α-amanitin. Such loss of toxicity may be due to a strongly reduced penetration rate into cells or by different pharmacokinetics of the cationic toxin. The low susceptibility of parenchymal cells to this compound encourages us to search for unknown specific cytotoxic activities.

(*8*) *Introduction of Functional Groups.* The smooth reaction by which long-chained alkyl bromides, even with unprotected functional groups, can be introduced into α-amanitin made it possible to also try to introduce spacer moieties which could be used to attach the toxins to proteins, solid supports, or reporter groups. For this purpose, a bivalent spacer was synthesized: ω-bromocapropylethylendiamine, with the terminal amino function protected by the *tert*-butyloxycarbonyl residue widely used in peptide chemistry.

The condensation of this complex alkyl bromide could be optimized to 46% yield, the highest in this series. The terminal amino function was deblocked by a 2-min reaction with trifluroacetic acid. The successful deblocking with the strong acid is particularly remarkable, because trifluoroacetic acid is a reagent converting bicyclic toxins of the phallotoxin and the amatoxin type into their monocyclic and inactive seco compounds. This reaction, however, needs several hours and was not detectable, even in traces, after the 2-min incubation. The spacer moiety introduced into α-aminitin is ten atoms long and contains an amide group. The polar region was assumed to be important to avoid nonspecific interaction of the spacer with lipophilic domains of proteins, as reported for long apolar hydorcarbon chains. In fact, we successfully used this spacer to introduce a fluorescent reporter group into α-amanitin. The compound was used for the study of the distribution of amatoxin-binding structures in PtK_1 cells (Wulf et al., 1980). Similarly, we conjugated the succinylated α-amanitin to several proteins, particularly fetuin, which were used as antigens (Faulstich et al., 1980). We then succeeded by means of this spacer to attach α-amanitin to solid supports. Using α-amanitin coupled to Sepharose 6B, we achieved in a one-step procedure the purification of the amatoxin-binding globulins from the serum of two rabbits (H. Faulstich, S. Zobeley, and H. Trischmann, unpublished experiments). Finally, by attaching the spacered α-amanitin to biotin, we developed a new affinity technique making use of two different biospecific interactions, amanitin with RNA polymerase II and biotin with avidin. With this technique, we were able to extract minute amounts of RNA polymerase II from crude homogenates of rat liver nuclei (H. Faulstich, A. C. Vaisius, and H. Trischmann, unpublished experiments).

Succinylation of the terminal amino group introduces a carboxylic group and increases the scale of modifying reactions.

In the succinylated form (**5n**), the toxin, if carefully dried, could be stored for some weeks at 4 °C.

(*9*) *Conclusions.* For the introduction of spacered functional groups, lipophilic residues, and radioactively labeled moieties into amatoxins, the phenolic hydroxy group of the indole nucleus was a good choice. According to the conformation model and to structure–activity investigations (Th. Wieland, C. Götzendörfer, G. Zanotti, and A. C. Vaisius, unpublished experiments), this part projects out of the cyclic peptide and, in contrast to the phallotoxins, does not seem to be part of the active site. Therefore, even derivatives with complex structures such as **5** (**o**, **n**, and **m**) still possess about 30% of the α-amanitin inhibition capacity. This residual activity is sufficiently high for the use of these compounds in biological systems. Also, the introduction of hydrocarbon chains into α-amanitin did not suppress the biological activity; in contrast, short chains reduced the inhibition capacity by only 30–40%. Such derivatives can be useful for introducing radioactive labels into amatoxins as well as for conferring lipophilic properties to α-amanitin and thereby enhancing the penetration rate into cells, possibly into cells other than those of liver and kidney usually attacked by the amatoxins.

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References

Buku, A., & Wieland, Th. (1975) *Justus Liebigs Ann. Chem.*, 1956.

Cochet-Meilhac, M., & Chambon, P. (1974) *Biochim. Biophys. Acta 353*, 160.

Cochet-Meilhac, M., Nuret, P., Courvalin, J. C., & Chambon, P. (1974) *Biochim. Biophys. Acta 353*, 185.

Faulstich, H., & Wieland, Th. (1971) *Eur. J. Biochem. 22*, 79.

Faulstich, H., & Trischmann, H. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 1395.

Faulstich, H., Trischmann, H., & Zobeley, S. (1980) in *Amanita Toxins and Poisoning* (Faulstich, H., Kommerell, B., & Wieland, Th., Eds.) p 37, Witzstrock Publishing Co., Baden-Baden and New York.

Geiger, R., & König, W. (1971) *Justus Liebigs Ann. Chem. 750*, 168.

Gowindan, M. V. (1969) Diploma Work, University of Heidelberg.

Greenleaf, A. L., & Bautz, E. K. F. (1975) *Eur. J. Biochem. 60*, 169.

Hencin, R. S., & Preston, J. F. (1979) *Mol. Pharmacol. 16*, 961.

Kostansek, E. C., Lipscomb, W. N., Yocum, R. R., & Thiessen, W. E. (1977) *J. Am. Chem. Soc. 99*, 1273.

Meilhac, M., Kedinger, C., Chambon, P., Faulstich, H., Gowindan, M. V., & Wieland, Th. (1970) *FEBS Lett. 9*, 258.

Morris, P. W., Venton, D. L., & Kelley, K. M. (1978) *Biochemistry 17*, 690.

Patel, D. J., Tonelli, A. E., Pfänder, P., Faulstich, H., & Wieland, Th. (1973) *J. Mol. Biol. 79*, 185.

Tonelli, A. E., Patel, D. J., Wieland, Th. & Faulstich, H. (1978) *Biopolymers 17*, 1973.

Trefouel, I., Trefouel, I., Nitti, F., & Bovet, D. (1935) *C. R. Seances Soc. Biol. Ses. Fil. 120*, 756.

Wieland, Th., & Boehringer, W. (1960) *Justus Liebigs Ann. Chem. 635*, 178–181.

Wieland, Th., & Fahrmeir, A. (1970) *Justus Liebigs Ann. Chem. 736*, 95.

Wieland, Th., & Faulstich, H. (1978) *CRC Crit. Rev. Biochem. 5*, 185.

Wieland, Th., & Faulstich, H. (1979) in *Frontiers in Bioorganic Chemistry and Molecular Biology* (Ovchinnikov, Y. A., & Kolosov, M. N., Eds.) p 97, Elsevier/North-Holland, Amsterdam.

Wieland, H., Hallermayer, R., & Zilg, W. (1941) *Justus Liebigs Ann. Chem. 548*, 1.

Wulf, E., Bautz, F. A., Wieland, Th., & Faulstich, H. (1980) *Exp. Cell Res. 130*, 415–420.